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(54) Title: BREAST CANCER RESISTANCE GENES, METHODS FOR THEIR DETECTION AND USES THEREOF

(57) Abstract

The present invention relates to the finding of genes associated with the development of estrogen independent malignant cell growth, particularly of breast cancer cells. The invention provides assay methods for the diagnosis or prognosis of malignant cell growth, which method comprises measuring the expression of one or more of the *BCAR1*, *BCAR2* or *BCAR3* genes in a biological sample from a patient. Measurement may be by immunological techniques or by hybridisation with nucleic acids, utilising novel sequences and antibodies of the invention.

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BREAST CANCER RESISTANCE GENES, METHODS FOR THEIR DETECTION AND USES THEREOF.

FIELD OF THE INVENTION

The invention relates to the clinical monitoring of cancer.

BACKGROUND OF THE INVENTION

Development of both normal and malignant breast tissue is regulated by growth factors and steroid hormones (Dickson and Lippman, 1995, Endocr. Rev., 16: 559-589), which interact with specific cellular receptors to initiate a complex signal cascade ultimately leading to cell proliferation and differentiation (Ullrich and Schlessinger, 1990, Cell, 61: 203-212; Mangelsdorf et al., 1995, Cell, 83: 835-839; Beato et al., 1995, Cell, 83: 851-857; Katzenellenbogen et al., 1996, Mol. Endocrinol., 10: 119-131). The important role of estrogens in epithelial cell growth has been clinically exploited to interfere with this signal cascade in breast tumors. Antagonists of estrogens, in particular tamoxifen, have been widely used in breast cancer treatment (Pritchard and Sutherland, 1989, Hematol. Oncol. Clin. North Amer., 3: 765-805; Santen et al., 1990, Endocr. Rev., 221-265) and are thought to compete with estrogen binding to the estrogen receptor (ER). Tamoxifen treatment of breast cancer has been shown to be effective in an adjuvant setting after primary surgery (Early Breast Cancer Trial Collab. Group, 1992, Lancet, 339: 1-15 and 71-85) and provides objective responses in half of the patients with advanced disease and ER-positive primary tumors (Pritchard and Sutherland, 1989, supra; Santen et al., 1990, supra; Horwitz, 1993, Breast Cancer Res. Treat., 26: 119-130). However, response duration in these patients is limited due to acquisition of tamoxifen resistance and tumor progression. Furthermore, half of the patients with ER-positive primary tumors won't respond to tamoxifen treatment (intrinsic resistance).

The causes for intrinsic and acquired tamoxifen resistance in ER-positive breast cancer are poorly understood and may involve altered pharmacology of the drug (Jordan and Murphy, 1990, Endocr. Rev., 11: 578-610; Osborne et al., 1991, J. Nat. Cancer Inst., 83: 1477-1482; Johnston et al., 1993, Lancet, 342: 1521-1522), alterations in the estrogen receptor structure and function (McGuire et al., 1991, Mol. Endocrinol., 5: 1571-1577; Karnik et al., 1994, Cancer Res., 54: 349-353; Roodi et al., 1995, J. Nat. Cancer Inst., 87:

446-451; Daffada et al., 1995, Cancer Res., 55: 288-293; Murphy et al., 1995, Clin. Cancer Res., 1: 155-159) and changes in the paracrine interactions with the stromal cells (Clarke et al., 1992, Crit. Rev. Oncol. Hematol., 12: 1-23). It has been speculated that genetic alterations in the tumor cells may contribute to development of acquired tamoxifen resistance and that these genes could be responsible for intrinsic resistance of ER-positive tumors (Dorssers et al., 1991, "Genetic mechanisms involved in progression to hormone independence of human breast cancer", in Mechanisms of progression to hormone-independent growth of breast and prostatic cancer, Berns, Romijn and Schroeder (eds.), pp. 169-182, Parthenon, Carnforth, UK; Clarke et al., 1993, Breast Cancer Res. Treat., 24: 227-239; Toi et al., 1993, Brit. J. Cancer, 68: 1088-1096; Van Agthoven et al., 1994, Mol. Endocrinol., 8: 1474-1483).

One object of the invention is to provide clinical testing of tissue to detect cells that are malignant and/or that exhibit hormone-independent proliferation, a feature of anti-cancer drug resistance in cancer patients.

Another object of the invention is to monitor the progression of cancers of estrogen-dependent tissues for the transition to antiestrogen insensitivity, which is indicated by detection of estrogen-independent tumor cells.

Another object of the invention relates to clinical assessment of breast cancer, wherein the detection of antiestrogen-resistant tumor cells is indicative of the progression to an aggressive cancer phenotype.

SUMMARY OF THE INVENTION

The present invention encompasses a method for the detection of a malignant cell, comprising measuring expression of one or more of the *BCAR1*, *BCAR2* and *BCAR3* genes in a biological sample, wherein detection of expression of one or more of these genes at a level in the sample that is elevated in comparison to that observed in normal tissue is indicative of a malignant cell.

As used herein, "malignant" refers to cancerous cell growth.

Preferably, the malignant cell so detected is endocrine-independent.

In a preferred embodiment, this method further comprises the step of detecting expression of one or more of the *BCAR1*, *BCAR2* and *BCAR3* genes at a level in the sample that is elevated in comparison to normal tissue, wherein the elevated expression is indicative

of a malignant cell.

As used herein, "elevated" is defined to mean an increase of at least 5-fold in the level of expression of a gene; preferably, such an increase is 15-fold the level of expression of a gene; most preferably, such an increase is 30- or greater fold the level of expression of a gene. Such an increase may also be 100- or greater fold the level of expression of a gene or 500- or greater fold the level of expression of a gene over that observed in normal control cells.

"Expression" is defined to mean the production of an mRNA transcript, or a portion or fragment of an mRNA transcript, of a gene or the production and/or modification of a protein, or a portion or fragment of a protein, encoded by a gene.

"Modification" is defined to mean post-translational cleavage, glycosylation, phosphorylation or dephosphorylation of a protein.

As used herein, "expression level" is defined to mean one or more of the rate or absolute level of transcription of the gene, stability of the mRNA transcript of the gene or of a portion or fragment of that transcript, rate and absolute level of translation the mRNA or of a portion or fragment of that mRNA and stability and native biological activity of the protein product or modified protein product of the mRNA or of a portion or fragment of that protein or modified protein.

As used herein, "modulation" is defined to mean elevation or depression of the expression level of a gene in cells of a sample being tested according to the invention relative to that observed in a sample of normal control cells.

A "tumor cell" is any cell that has undergone one or more rounds of cell division that take place outside the course of normal growth or biological function (for example, that of a stem cell) or that are not required for routine cell replacement or wound healing in the tissue of which that cell is a part. The terms "tumor cell" and "malignant cell" are used synonymously for the purposes of the present invention.

"Normal tissue" is defined as being one or more cells that are not tumor cells and that do not possess a malignant phenotype (that is, that do not display cancerous cell growth), as defined above.

The term "biological sample", as used herein, is defined as any sample of tissue or bodily fluid, such as blood or a fraction thereof, milk, lymphatic fluid, vaginal fluid or fluid of the uterine lining, obtained from a patient that comprises one or more tumor cells, as

defined above.

It is preferred that the malignant cell detected by the methods of the present invention is antiestrogen-resistant.

As used herein, "estrogen-dependent" or "estrogen-sensitive" refer to a signalling pathway positively controlling proliferation of certain cell types which is activated by binding of estrogen to the estrogen receptor (ER) of these cells. Additionally, these terms refer to cells regulated by the pathway.

"Estrogen-independent" refers to a pattern of cell proliferation that is sustained for more than one round of division in the absence of estrogen, and preferably two or more- or three or more rounds of division in the absence of estrogen, as well as to cells that exhibit that pattern.

"Antiestrogen" is defined herein as an antagonist of the estrogen-dependent cell proliferative pathway. "Antagonist" refers to a substance capable of blocking the estrogen-dependent cell-proliferative pathway by competing with estrogen for binding to the ER, inactivating the ER, inactivating components of the pathway downstream of the estrogen/ER complex or of activating a pathway that inhibits estrogen-dependent cell proliferation. Examples of antiestrogen compounds include tamoxifen (4-hydroxy-tamoxifen or 4-OH-TAM) and ICI 182,780.

"Antiestrogen-resistant" refers to a pattern of cell proliferation that is sustained for more than one round of division in the presence of an antiestrogen compound, whether or not estrogen is present, as well as to cells that exhibit such a pattern.

It is preferred that the normal tissue is of the same tissue type from which the malignant cell has arisen.

It is preferred that the biological sample comprises tumor cells, more preferably tumor cells that have arisen from breast tissue.

It is additionally preferred that the measuring of a gene expression level comprises contacting the protein of the biological sample with an antibody directed at the protein product of the gene and performing a detection step for the antibody complexed to that product or to a portion or a fragment thereof, more preferred that the measuring comprises an ELISA (enzyme-linked immunosobent assay), a Western blot or most preferably immunostaining of fixed cells of the sample being tested according to the invention.

It is contemplated that the measuring of a gene expression level is of nucleic acid of

the biological sample, preferably mRNA.

Preferably, the measuring comprises performing hybridization of a labelled nucleic acid probe complementary to sequences of the gene to the mRNA and a step to detect hybridized complexes of the probe to the mRNA product of that gene or to a portion or a fragment thereof.

Preferably, the hybridization is performed on a Northern blot.

The present invention also encompasses a method of detecting a malignant cell, comprising measuring the expression of a panel of genes comprising one or more of a first gene of *BCAR1*, *BCAR2*, and *BCAR3* in combination with one or more of a second gene of *ER*, *EGFR*, *PR*, *PS2*, *RAS*, *ILGF-II*, *HER2/NEU*, *FGF-4*, *TGFβ1* and *Cyclin D1* in a biological sample from a patient and comparing the ratio of expression levels of these genes relative to one another in the sample to the ratio of expression levels of these first and second genes observed in normal tissue, wherein detection of a difference between the ratios of levels of expression of any two first and second genes of the panel in the biological sample and in the normal tissue is indicative of a malignant cell.

It is preferred that the malignant cell so detected is endocrine-independent.

Preferably, such a panel of genes comprises one or more of a first gene of *BCAR1*, *BCAR2* and *BCAR3* measured against one or more of a second gene of *RAS*, *insulin-like growth factor II*, *FGF-4*, *TGFβ1* and *cyclin D1*; more preferably, such a panel of genes comprises one or more of a first gene of *BCAR1*, *BCAR2* and *BCAR3* measured against one or both of a second gene of *EGFR* and *HER2/NEU*; most preferably, such a panel of genes comprises one or more of a first gene of *BCAR1*, *BCAR2* and *BCAR3* measured against one or more of a second gene of *ER*, *PR* and *PS2*.

Preferably, the method comprises the further step of obtaining a result wherein the ratio of expression levels measured for a pair of first and second genes selected from the panel of genes is elevated in a cell of the sample is in comparison to that observed in normal tissue, wherein an elevated ratio is indicative of a malignant cell.

As used herein, "elevated" is in this context defined to mean an increase of a least 2-fold in the ratio of expression levels of a first and second gene; preferably, such an increase is at least 10-fold in the ratio of expression levels of a first and second gene or is at least 20-fold in the ratio of expression levels of a first and second gene; more preferably, such an increase is at least 100-fold in the ratio of expression levels of a first and second gene or is at least

200-fold in the ratio of expression levels of a first and second gene; most preferably, such an increase is at least 1,000-fold in the ratio of expression levels of a first and second gene or is at least 10,000-fold in the ratio of expression levels of a first and second gene in cells of a sample being tested according to the invention relative to that observed in a sample of normal control cells.

It is preferred that the malignant cell so detected is antiestrogen resistant.

Preferably, the expression level measured for one or more of the *BCAR1*, *BCAR2*, and *BCAR3* genes is elevated.

It is preferred that the normal tissue is of the same tissue type from which the malignant cell has arisen.

Preferably, the biological sample comprises tumor cells, more preferably, the tumor cells have arisen from breast tissue.

The invention also encompasses a method for monitoring the malignancy of tumor cell growth, comprising measuring the level of expression of one or more of the *BCAR1*, *BCAR2* and *BCAR3* genes in a sample of tumor cells from a patient, wherein expression of one or more of these genes at a level in a cell of the sample that is elevated in comparison to that observed in normal tissue is indicative of progression to malignant tumor cell growth.

It is preferred that the malignant tumor cell growth so monitored is endocrine-independent, more preferably, the malignant tumor cell growth is antiestrogen-resistant.

In a preferred embodiment, this method further comprises the step of detecting expression of one or more of the *BCAR1*, *BCAR2* and *BCAR3* genes at a level in the sample that is elevated in comparison to normal tissue, wherein the elevated expression is indicative of malignant cell growth.

Preferably, the normal tissue is of the same tissue type from which the tumor cells have arisen.

It is additionally preferred that the tumor cells so monitored have arisen from breast tissue.

The invention also encompasses a method for monitoring the malignancy of tumor cell growth, comprising measuring the expression of a panel of genes comprising one or more of a first gene of *BCAR1*, *BCAR2*, and *BCAR3* in combination with one or more of a second gene of *ER*, *EGFR*, *PR*, *PS2*, *RAS*, *ILGF-II*, *HER2/NEU*, *FGF-4*, *TGFβ1* and *Cyclin D1* in a sample of tumor cells from a patient and comparing the ratio of expression levels of these

first and second genes in the sample to the ratio of expression levels of these first and second genes observed in normal tissue, wherein detection of a difference between the ratio of levels of expression of any two first and second genes of the panel in the tumor cells in comparison to that observed in normal tissue is indicative of progression to estrogen-independent tumor cell growth.

Preferably, such a panel of genes comprises one or more of a first gene of *BCAR1*, *BCAR2* and *BCAR3* measured against one or more of a second gene of *RAS*, *insulin-like growth factor II*, *FGF-4*, *TGFβ1* and *cyclin D1*; more preferably, such a panel of genes comprises one or more of a first gene of *BCAR1*, *BCAR2* and *BCAR3* measured against one or both of a second gene of *EGFR* and *HER2/NEU*; most preferably, such a panel of genes comprises one or more of a first gene of *BCAR1*, *BCAR2* and *BCAR3* measured against one or more of a second gene of *ER*, *PR* and *PS2*.

Preferably, the method comprises the further step of obtaining a result wherein the ratio of expression levels measured for a pair of first and second genes of the panel of genes is elevated in a cell of the sample of tumor cells in comparison to that observed in normal tissue, wherein an elevated ratio is indicative of estrogen-independent tumor cell growth.

It is preferred that the malignant tumor cell growth so monitored is endocrine-independent, more preferred that it is antiestrogen resistant.

In a preferred embodiment, the expression level measured for one or more of the *BCAR1*, *BCAR2*, and *BCAR3* genes in the sample of tumor cells is elevated in comparison to that observed in normal tissue.

Preferably, the normal tissue is of the same tissue type from which the tumor has arisen.

It is also preferred that the tumor cells have arisen from breast tissue.

The present invention also includes a method for detecting an estrogen-independent breast tumor cell, comprising measuring the expression of one or more of the *BCAR1*, *BCAR2*, and *BCAR3* genes in a biological sample from a patient, wherein detection of expression of one or more of these genes at a level in the biological sample that is elevated in comparison to that observed in normal breast tissue is indicative of an estrogen-independent breast tumor cell.

It is preferred that the method comprises the step of obtaining a result wherein one or more of the *BCAR1*, *BCAR2* and *BCAR3* genes is expressed at a level in the biological

sample that is elevated in comparison to that observed in normal breast tissue, wherein elevated expression is indicative of an estrogen-independent breast tumor cell.

It is preferred that the breast tumor cell so detected is antiestrogen resistant.

It is additionally preferred that the biological sample comprises breast tumor cells.

Also encompassed by the present invention is a method of detecting an estrogen-independent breast tumor cell, comprising measuring the expression of a panel of genes comprising one or more of a first gene of *BCAR1*, *BCAR2* and *BCAR3* in combination with one or more of a second gene of *ER*, *EGFR*, *PR*, *PS2*, *RAS*, *ILGF-II*, *HER2/NEU*, *FGF-4*, *TGFβ1* and *Cyclin D1* in a biological sample from a patient and comparing the ratio of expression levels of these first and second genes in the sample to the ratio of expression levels of these first and second genes observed in normal breast tissue, wherein detection of a difference in the ratios of levels of expression of any two genes of the panel in the biological sample and in normal breast tissue is indicative of an estrogen-independent breast tumor cell.

Preferably, such a panel of genes comprises one or more of a first gene of *BCAR1*, *BCAR2* and *BCAR3* measured against one or more of a second gene of *RAS*, *insulin-like growth factor II*, *FGF-4*, *TGFβ1* and *cyclin D1*; more preferably, such a panel of genes comprises one or more of a first gene of *BCAR1*, *BCAR2* and *BCAR3* measured against one or both of a second gene of *EGFR* and *HER2/NEU*; most preferably, such a panel of genes comprises one or more of a first gene of *BCAR1*, *BCAR2* and *BCAR3* measured against one or more of a second gene of *ER*, *PR* and *PS2*.

It is preferred that the method further comprises the step of obtaining a result wherein the ratio of expression levels measured for a pair of first and second genes of the panel is elevated in a cell of the sample in comparison to that observed in normal breast tissue, wherein an elevated ratio is indicative of an estrogen-independent breast tumor cell.

Preferably, the expression level measured for one or more of the *BCAR1*, *BCAR2* and *BCAR3* genes is elevated.

It is preferred that the biological sample comprises breast tumor cells.

It is also preferred that the estrogen-independent breast tumor cell so detected is antiestrogen resistant.

The present invention also encompasses a method for monitoring the estrogen dependence of breast tumor cell growth, comprising measuring the level of expression of one or more of the *BCAR1*, *BCAR2* and *BCAR3* genes in a sample of breast tumor cells from a

patient, wherein expression of one or more of these genes at a level in a cell of the sample that is elevated in comparison to that observed in normal breast tissue is indicative of progression to estrogen-independent breast tumor cell growth.

It is preferred that the breast tumor cell growth so monitored is antiestrogen-resistant.

Also disclosed herein is a method for monitoring the estrogen dependence of breast tumor cell growth, comprising measuring the expression of a panel of genes comprising one or more of a first gene of *BCAR1*, *BCAR2* and *BCAR3* in combination with one or more of a second gene of *ER*, *EGFR*, *PR*, *PS2*, *RAS*, *ILGF-II*, *HER2/NEU*, *FGF-4*, *TGFβ1* and *Cyclin D1* in a sample of breast tumor cells from a patient and comparing the ratio of expression levels of these first and second genes relative to one another in the sample to the ratio of expression levels of these first and second genes observed in normal breast tissue, wherein detection of a difference in the ratio of levels of expression of any two first and second genes of the panel in the biological sample and normal breast tissue is indicative of progression to estrogen-independent breast tumor cell growth.

Preferably, such a panel of genes comprises one or more of a first gene of *BCAR1*, *BCAR2* and *BCAR3* measured against one or more of a second gene of *RAS*, *insulin-like growth factor II*, *FGF-4*, *TGFβ1* and *cyclin D1*; more preferably, such a panel of genes comprises one or more of a first gene of *BCAR1*, *BCAR2* and *BCAR3* measured against one or both of a second gene of *EGFR* and *HER2/NEU*; most preferably, such a panel of genes comprises one or more of a first gene of *BCAR1*, *BCAR2* and *BCAR3* measured against one or more of a second gene of *ER*, *PR* and *PS2*.

It is preferred that the method comprises the further step of obtaining a result wherein the ratio of expression levels measured for a pair of first and second genes of the panel of genes is elevated in a cell of the sample in comparison to that observed in normal breast tissue, wherein an elevated ratio is indicative of estrogen-independent breast tumor cell growth.

Preferably, the expression level measured for one or more of the *BCAR1*, *BCAR2* and *BCAR3* genes is elevated.

It is additionally preferred that the breast tumor cell growth so monitored is anti-estrogen resistant.

The present invention also encompasses the DNA sequence presented in SEQ ID NO: 1, the protein sequence presented in SEQ ID NO: 2, the DNA sequence presented in SEQ ID

NO: 3, the protein sequence presented in SEQ ID NO: 4, the DNA sequences presented in SEQ ID NO: 7-19, the DNA sequence presented in SEQ ID NO: 20, the protein sequence presented in SEQ ID NO: 21, the DNA sequence presented in SEQ ID NO: 22, the protein sequence presented in SEQ ID NO: 23, the DNA sequence presented in SEQ ID NO: 24 and the protein sequence presented in SEQ ID NO: 25. The invention also encompasses fragments and variants of said sequences as defined herein.

Measurement of the level of expression of *BCAR1* may comprise the use of a nucleic acid probe whose sequence is specific for the nucleotide sequence of SEQ ID NO:1 or its complement, particularly to nucleotides 122 to 2731 of SEQ ID NO:1. Likewise, measurement of the level of expression of *BCAR2* may comprise the use of a probe whose sequence is specific for SEQ ID NO:20, 22 or 24 or their complements, particularly for specific to nucleotides 570-4169 of SEQ ID NO:20. Further, measurement of the level of expression of *BCAR3* may comprise the use of a probe whose sequence is specific for SEQ ID NO:3, particularly nucleotides 39 to 3042 or nucleotides 99 to 2573 or its complement.

By "specific", it is meant that the probe is one which is capable of hybridizing to its target *BCAR1, 2* or *3* sequence or its complement in a human cell under conditions where said probe does not hybridize to other nucleic acid sequence normally found in a human cell.

For short probes, for example of 10 to 50 nucleotides, the precise conditions will depend upon the precise sequence and GC content of the probe, and such conditions may be determined experimentally using routine skill in the art. For longer probes, such conditions are typically high stringency conditions which may be applied to the probe following annealing to a *BCAR1, 2* or *3* target sequence under annealing conditions. An example of a low stringency annealing condition for nucleic acid bound to a solid phase is, for example, 6xSSC at 55°C. This may be followed by washing at reduced SSC and/or higher temperature, for example at 0.2xSSC at 45°C, and increasing the hybridisation temperature incrementally to determine hybridisation conditions which allow the probe to hybridise to the target *BCAR1, 2* or *3* sequence but not other sequences found in a normal human cell.

Preferred nucleic acid sequences which may be used as probes are those based upon the sequences of SEQ ID NOs:1, 3, 20, 22 or 24, or their complements, or sequences which have high homology to these sequence, for example which are at least 70%, preferably at least 80% such as at least 90% or event at least 95% homologous to said sequences or their complements. Homology (also referred to as "identity") may be determined by suitable

algorithms. For example, the percentage homology (also referred to as identity) of DNA sequences can be calculated using commercially available algorithms, such as Lasergene software from DNASTAR Inc or the algorithm GAP (Genetics Computer Group, Madison, WI). GAP uses the Needleman and Wunsch algorithm to align two complete sequences that maximizes the number of matches and minimizes the number of gaps. Generally, the default parameters are used, with a gap creation penalty = 12 and gap extension penalty = 4.

Use of either of the terms "homology" and "homologous" herein does not imply any necessary evolutionary relationship between compared sequences, in keeping for example with standard use of terms such as "homologous recombination" which merely requires that two nucleotide sequences are sufficiently similar to recombine under the appropriate conditions.

Thus probes, which form a further aspect of the invention, may be a sequence of at least 10 nucleotides, for example at least 12, 15, 18, 21, 24, 30 or 50 nucleotides of a sequence which has at least 70% homology to SEQ ID NOs:1, 3, 20, 22 or 24. Probes may also be based on much larger fragments of said sequences or sequences highly homologous thereto, for example fragments of from 50 to 3205, such as from 50 to 1000 or 100 to 500 nucleotides. Short probes will usually be generated by oligonucleotide synthesis, and may directed to either strand of the target sequences. Longer probes may be single or double stranded (e.g. generated by transcription from a T7 promoter or by restriction digest of a *BCAR1, 2* or *3* clone respectively) and where single, may also be directed to either strand. Where mRNA is to be detected, at least one probe will be directed to the coding strand of the target *BCAR* sequence, although where detection is by PCR or other amplification methods, a pair of probes will be used, one of which will be directed to the complementary strand.

Nucleic acid includes DNA (including both genomic and cDNA)and RNA, and also synthetic nucleic acids, such as those with modified backbone structures intended to improve stability of the nucleic acid in a cell. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or lifespan of polynucleotides of the invention. Where nucleic acid according to the invention includes

RNA, reference to the sequences shown in the accompanying listings should be construed as reference to the RNA equivalent, with U substituted for T.

Nucleic acid of the invention may be single or double stranded polynucleotides. Single stranded nucleic acids of the invention include anti-sense nucleic acids. Such antisense nucleic acids may be stabilized as described above. They may be in the form of a composition comprising said anti-sense nucleic acids and a carrier or diluent. The sizes of said nucleic acids may be from 10 to 50 nucleotides, as describe herein for probes. Antisense oligonucleotides may be designed to hybridise to the complementary sequence of nucleic acid, pre-mRNA or mature mRNA, interfering with the production of polypeptide encoded by a given DNA sequence so that its expression is reduce or prevented altogether. The construction of antisense sequences and their use is described in Peyman and Ulman, Chemical Reviews, 90:543-584, (1990), Crooke, Ann. Rev. Pharmacol. Toxicol., 32:329-376, (1992), and Zamecnik and Stephenson, P.N.A.S, 75:280-284, (1974).

A nucleic acid of the invention may be carried in a vector, optionally operably linked to a control sequence which is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector.

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage phagemid or baculoviral, cosmids, YACs, BACs, or PACs as appropriate. Vectors may also be adenovirus, adeno-associated virus, retrovirus (such as HIV or MLV) or alpha virus vectors. The latter may be used to infect human or mammalian cells for the study of the function of the *BCAR1, 2* or *3* genes, for example their cellular localization. Such vectors may also be suitable for gene therapy, for example by expression of anti-sense gene sequences (which when expressed from vectors may be longer than those of anti-sense oligonucleotides discussed above) or of portions of a *BCAR1, 2 or* 3 genes or variants thereof to provide a polypeptide product which antagonizes the function of the full length protein, for example in the form of a dominant negative product.

The vectors may be provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a

mammalian vector. Vectors may be used *in vitro*, for example for the production of RNA or used to transfect or transform a host cell. Suitable host cells include bacteria, eukaryotic cells such as mammalian and yeast, and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, COS cells and many others.

Promoters and other expression regulation signals may be selected to be compatible with the host cell for which the expression vector is designed. For example, yeast promoters include S. cerevisiae GAL4 and ADH promoters, S. pombe nmt1 and adh promoter. Mammalian promoters include the metallothionein promoter which is can be included in response to heavy metals such as cadmium. Viral promoters such as the SV40 large T antigen promoter or adenovirus promoters may also be used. All these promoters are readily available in the art.

For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley & Sons, 1992.

A further embodiment of the invention provides host cells transformed or transfected with the vectors for the replication and expression of polynucleotides of the invention. The cells will be chosen to be compatible with the said vector and may for example be bacterial, yeast, insect or mammalian. Such host cells may be used to make the polypeptides of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:21, SEQ ID NO:23 or SEQ ID NO:25, or variants thereof which share at least 70%, preferably at least 80%, more preferably at least 90% and most preferably at least 95% identity thereto and fragments thereof, preferably those which contain an epitope. Such fragments are preferably at least 10, preferably at least 15, more preferably at least 20, 30 or 40 such as at least 50 amino acids in size (apart from SEQ ID NO:23 and SEQ ID NO:25 for which fragments will be a maximum of less than 20 and 12 respectively). Such polypeptides, which may be isolated, form a further aspect of the invention.

The invention disclosed herein encompasses a kit for the detection of the mRNA product of the *BCAR1* gene, comprising a nucleic acid probe at least 10 nucleotides in length

that is specific for sequences encompassed by SEQ ID NO: 1 and packaging materials therefor.

The invention further encompasses a kit for the detection of the mRNA product of the *BCAR2* gene, comprising a nucleic acid probe at least 10 nucleotides in length that is specific for sequences encompassed by SEQ ID NO: 20, 22 or 24 and packaging materials therefor.

The invention additionally encompasses a kit for the detection of the mRNA product of the *BCAR3* gene, comprising a nucleic acid probe at least 10 nucleotides in length that is specific for sequences encompassed by SEQ ID NO: 3 and packaging materials therefor.

Also claimed is a planar array of RNA samples derived from breast tissue affixed to a solid support matrix and probed with a labelled nucleic acid probe at least 10 nucleotides in length that is specific for sequences encompassed by SEQ ID NO: 1, 3, 20, 22 or 24 as well as an autoradiographic film that has been exposed to the array for a time and under conditions sufficient to permit detection of bound probe, and a digital, photographic, xerographic or chart record of the array that contains evidence of bound probe that is labelled luminescently, radioactively or with a visible chemical precipitate on the support matrix.

Preferably, the planar array of RNA samples is one of a Northern blot or an RNA dot blot.

The present invention includes an autoradiographic film that has been exposed to a sample of cells to which a labelled nucleic acid probe at least 10 nucleotides in length that is specific for sequences encompassed by SEQ ID NO: 1, 3, 20, 22 or 24 has been hybridized for a time and under conditions sufficient to permit detection of bound probe.

The provision of the novel polypeptides of the invention allows for the production of novel antibodies which are able to bind to these polypeptides in a specific manner. Such an antibody may be specific in the sense of being able to distinguish between the polypeptide it is able to bind and other polypeptides of the same species for which it has no or substantially no binding affinity (e.g. a binding affinity of at least about 1000x worse). Specific antibodies bind an epitope on the molecule which is either not present or is not accessible on other molecules. Antibodies according to the present invention may be specific for the wild-type polypeptide, or variants thereof.

Although in the case of BCAR1, we have found that known antibodies from other species cross-react to the BCAR protein, such antibodies, although not claimed as such, are still specific for the BCAR1 protein within a human cell. Furthermore, the sequence

differences between BCAR1 and the p130cas proteins illustrated in Figure 2 may give rise to novel epitopes to which novel antibodies which form part of the present invention.

Antibodies are also useful in purifying the polypeptide or polypeptides to which they bind, e.g. following production by recombinant expression from encoding nucleic acid.

Preferred antibodies according to the invention are isolated, in the sense of being free from contaminants such as antibodies able to bind other polypeptides and/or free of serum components. Monoclonal antibodies are preferred for some purposes, though polyclonal antibodies are within the scope of the present invention.

Antibodies may be obtained using techniques which are standard in the art. Methods of producing antibodies include immunising a mammal (e.g. mouse, rat, rabbit) with a BCAR protein or fragment thereof and recovering polyclonal antibodies or using cells of the immunized mammal to make monoclonal antibodies. Alternatively, an antibody specific for a protein may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047.

The term "antibody" should be construed as covering any binding substance having a binding domain with the required specificity. Thus the invention covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including synthetic molecules and molecules whose shape mimics that of an antibody enabling it to bind an antigen or epitope.

Example antibody fragments, capable of binding an antigen or other binding partner are the Fab fragment consisting of the VL, VH, Cl and CH1 domains; the Fd fragment consisting of the VH and CH1 domains; the Fv fragment consisting of the VL and VH domains of a single arm of an antibody; the dAb fragment which consists of a VH domain; isolated CDR regions and F(ab')2 fragments, a bivalent fragment including two Fab fragments linked by a disulphide bridge at the hinge region. Single chain Fv fragments are also included.

Humanized antibodies in which CDRs from a non-human source are grafted onto human framework regions, typically with the alteration of some of the framework amino acid residues, to provide antibodies which are less immunogenic than the parent non-human antibodies, are also included within the present invention.

Hybridomas capable of producing antibody with desired binding characteristics are within the scope of the present invention, as are host cells, eukaryotic or prokaryotic, containing nucleic acid encoding antibodies (including antibody fragments) and capable of their expression.

The reactivities of antibodies on a sample may be determined by any appropriate means. Tagging with individual reporter molecules is one possibility. The reporter molecules may directly or indirectly generate detectable, and preferably measurable, signals. The linkage of reporter molecules may be directly or indirectly, covalently, e.g. via a peptide bond or non-covalently.

An antibody may be provided in a kit, which may include instructions for use of the antibody, e.g. in determining the presence of a particular substance in a test sample. One or more other reagents may be included, such as labelling molecules, buffer solutions, elutants and so on. Reagents may be provided within containers which protect them from the external environment, such as a sealed vial.

Also encompassed by the present invention is an autoradiographic film that has been exposed to a sample of cells to which is bound a labelled primary antibody directed against one or more of the proteins BCAR1, BCAR2 and BCAR3, for a time and under conditions sufficient to permit detection of the bound, labelled antibody, as well as a digital, photographic, xerographic or chart record of the cells that documents the presence on the cells of bound, labelled antibody.

The present invention further encompasses an autoradiographic film that has been exposed to a sample of cells to which is bound a primary antibody directed against one of more of the proteins BCAR1, BCAR2 and BCAR3 and a labelled secondary antibody directed against the primary antibody for a time and under conditions sufficient to permit detection of the labelled secondary antibody, as well as a digital, photographic, xerographic or chart record of the cells that documents the presence on the cells of bound, labelled secondary antibody.

The present invention encompasses an autoradiographic, digital, photographic, xerographic or chart record of a planar array of proteins on the surface of a solid support matrix to which is bound a primary antibody directed against one or more of the proteins BCAR1, BCAR2 and BCAR3 and a labelled secondary antibody directed against the primary antibody that documents the presence on the matrix of bound, labelled secondary antibody.

The invention further provides ribozymes which comprise a nucleic acid sequence of the invention. The invention further comprises the use of such ribozymes in a method of cleaving mRNA expressed by *BCAR1, 2* or *3* genes, the method being performed in a cell free system, *in vitro* or *in vivo*. The construction of ribozymes and their use in described in for instance Gibson and Shillitoe, Molecular Biotechnology 7(2): 125-137, (1997).

In a further aspect, the invention provides a method of producing a transgenic non-human mammal having a predisposition to tumors (particularly mammary or ovarian tumors), said method comprising incorporating a *BCAR1, 2* or *3* encoding nucleic acid (or fragment thereof) operably linked to a promoter capable of bringing about enhanced expression of said gene in said mammal. The invention also extends to a transgenic non-human mammal whose germ cells and somatic cells carry such a construct as a result of chromosomal incorporation into the mammal genome, or into the genome of an ancestor of said mammal. The promoter may be a constitutive promoter which brings about gene expression at a higher level than found for BCAR genes in normal tissue, or may be an inducible promoter, such as a metallothionein promoter which responds to hormones or metal ions. It may also be a viral promoter such as a retroviral LTR. It may also be a promoter expressed in lactation. For a description of such a promoter, see Wall, Transgenic Research, Vol.5(1): 67-72, (1996) and Niemann, Journal of Animal Breeding and Genetics, Vol.113(4-5): 437-444, (1996) which is incorporated herein by reference.

The production of transgenic animals may be achieved in a variety of ways. A typical strategy is to use targeted homologous recombination to replace, modify or delete a wild-type target gene in an embryonic stem (ES) cell. An targeting vector is introduced into ES cells by electroporation, lipofection or microinjection. In a few ES cells, the targeting vector pairs with the cognate chromosomal DNA sequence and transfers the desired mutation carried by the vector into the genome by homologous recombination. Screening or enrichment procedures are used to identify the transfected cells, and a transfected cell is cloned and maintained as a pure population. Next, the altered ES cells are injected into the blastocyst of a preimplantation mouse embryo or alternatively an aggregation chimera is prepared in which the ES cells are placed between two blastocysts which, with the ES cells, merge to form a single chimeric blastocyst. The chimeric blastocyst is surgically transferred into the uterus of a foster mother where the development is allowed to progress to term. The resulting animal will be a chimera of normal and donor cells. Typically the donor cells will be from a animal

with a clearly distinguishable phenotype such as skin colour, so that the chimeric progeny is easily identified. The progeny is then bred and its descendants cross-bred, giving rise to heterozygotes and homozygotes for the targeted mutation. The production of transgenic animals is described further by Capecchi, M, R., 1989, Science 244; 1288-1292; Valancius and Smithies, 1991, Mol. Cell. Biol. 11; 1402-1408; and Hasty et al, 1991, Nature 350; 243-246, the disclosures of which are incorporated herein by reference.

Transgenic targeting techniques may also be used to delete a target *BCAR* gene. Methods of targeted gene deletion are described by Brenner et al, WO94/21787 the disclosure of which is incorporated herein by reference.

The invention extends to transgenic non-human mammals obtainable by such methods and to their progeny. Such mammals may be homozygous or heterozygous. Such mammals include mice, rodents, rabbits, sheep, goats, pigs.

Transgenic non-human mammals may be used for experimental purposes in studying the development or progression of tumors, and in the development of therapies designed to alleviate the symptoms or progression of tumors, particularly estrogen resistant breast tumors caused by a defect in the structure or expression level of a *BCAR1,* 2 or 3 gene. By "experimental" it is meant permissible for use in animal experimentation or testing purposes under prevailing legislation applicable to the research facility where such experimentation occurs.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 displays Northern hybridization of poly(A)$^+$ RNA from ZR-75-1 cells, antiestrogen-resistant ZR-75-1 derivatives cell lines and other breast cancer cell lines to detect the *BCAR1* transcript.

Figure 2 presents a protein sequence comparison between human BCAR1 [SEQ ID NO: 2] and the p130cas proteins of the rat [SEQ ID NO: 5] and the mouse [SEQ ID NO: 6].

Figure 3 diagrams the growth kinetics of transfected ZR-75-1 cells expressing the *BCAR1* cDNA (4A-12), an empty expression vector (2A-4 and 2A-5) or in a cell-fusion-mediated hybrid (D436) containing the retrovirally-mutated *BCAR1* locus, in medium containing 4-hydroxy-tamoxifen.

Figure 4 presents the identification of retroviral integration loci in somatic cell hybrids.

Figure 5 shows photomicrographs of antiestrogen-sensitive (Fig. 5A) and -resistant (Fig. 5B) somatic cell hybrids under 4-hydroxy-tamoxifen selection.

Figure 6 contains a graphic representation of the proliferative capacity of cell lines in the presence of 4-hydroxy-tamoxifen.

Figure 7 depicts antiestrogen-resistant proliferation of somatic cell hybrids. Figure 7A, Figure 7B and Figure 7C together display a single data set that has been divided in three in the interest of visual clarity.

Figure 8 depicts the proliferative capacity of somatic cell hybrids.

Figure 9 shows identification of the *BCAR2* locus on Southern blots.

Figure 10 presents genomic analysis of the *BCAR3* locus.

Figure 11 shows the effect of successful transfer of the BCAR3 locus in a somatic cell hybrid.

Figure 12 presents mRNA expression of *BCAR3*.

Figure 13 presents a schematic representation of the *BCAR3* gene.

Figure 14 displays the expression of *BCAR3* mRNA in various normal tissues.

Figure 15 shows a comparison of *in vitro* translated full-length (B3) and shortened (B3S) BCAR3 proteins.

Figure 16 depicts the effect of *BCAR3* expression on antiestrogen-resistance.

Figure 17 presents genomic DNA sequences adjacent to the *BCAR1* exons.

Figure 18 represents analysis of BCAR1 protein levels in primary breast tumors, and demonstrates an association of detection of BCAR1 protein with an increased rate of relapse.

DESCRIPTION OF THE INVENTION

The present invention encompasses methods and compositions for detection of malignant cells. In particular methods for detecting such cells by measuring the level of one or more of three genes, designated *Breast Cancer Antiestrogen Resistance 1 (BCAR1), BCAR2* and *BCAR3*, that are herein demonstrated to be abnormally expressed in cancers of estrogen-dependent cell types.

Example 1 describes the cloning of *BCAR1*.

Example 2 describes the identification of *BCAR2* and the cloning of a candidate *BCAR2* gene.

Example 3 describes the identification and cloning of *BCAR3*.

Example 4 describes the use of any of *BCAR1*, *BCAR2* and *BCAR3* to detect malignant cells.

Example 5 describes the use of a panel of genes comprising *BCAR1*, *BCAR2* and *BCAR3* in conjunction with other cell markers to detect malignant cells.

Example 6 describes immunohistochemical detection of the BCAR1, BCAR2 and BCAR3 or other proteins in tumor biopsy tissue.

Example 7 provides a representative diagnostic analysis according to the invention in which BCAR1 protein is detected in breast cancer tissue samples, but not in normal breast tissue samples, and is correlated with the rate of relapse in breast cancer patients and failure of response to antiestrogen therapy.

METHODS APPLICABLE IN THE PRESENT INVENTION

Methods applicable to making and using the invention, as described in detail in the following Examples, comprise standard molecular and biochemical techniques, such as bacterial and yeast cell culture, nucleic acid library construction and screening, DNA cloning and sequencing, the polymerase chain reaction (PCR), Southern, Northern and Western analysis and mammalian cell culture and transfection techniques. Also applicable may be histological methods such as *in situ* mRNA hybridization or immunocytochemistry, both by protocols well known in the art.

EXAMPLE 1

Identification of unknown, dominantly acting gene functions may be achieved by random transfection of genomic DNA or cDNA, somatic cell fusion or insertional mutagenesis. Retrovirus-mediated insertional mutagenesis (Goff, 1987, Methods Enzymol., 152: 469-481) has proven to be very powerful in identifying various genes contributing to rodent tumorigenesis *in vivo* (reviewed by Kung et al., 1991, Curr. Top. Microbiol. Immunol., 171: 1-25; Jonkers and Berns, 1996, Biochim. biophys. Acta Rev. Cancer, 1287: 29-57) and to *in vitro* invasion of mouse lymphoma cells (Habets et al., 1994, Cell, 77: 537-549) and progression to hormone independence of human breast cancer cells (Dorssers et al., 1993, Mol. Endocrinol., 870-878). Retrovirus integration may activate genes in its environment by promoter or enhancer insertion or modulate gene function by truncation of the transcript. The occurrence of a retroviral genome in virtually identical sites of the cellular genome of independently-derived cell lines having the selected phenotype (so-called common integration sites), is a very strong suggestion for the presence of the responsible

gene in the vicinity of the integrated provirus (Goff, 1987, supra; Jonkers and Berns, 1996, supra).

An *in vitro* screen for changes in cell growth phenotype (Dorssers et al., 1993, supra) employed defective retroviral insertional mutagenesis of breast cancer cell line ZR-75-1, which is strictly estrogen-dependent for proliferation and can be fully inhibited by antiestrogens (Van Agthoven et al., 1992, Cancer Res., 52: 5082-5088; Dorssers et al., 1993, supra). Because primary breast tumor cells are difficult to culture in large quantities for prolonged times, this cell line was selected as a model for hormone-dependent breast cancer. The screen yielded numerous estrogen-independent clonal isolates, each bearing one or more viral integrants. In the present application, we demonstrate that viral insertions at three such loci are individually capable of inducing estrogen independence (and, consequently, antiestrogen resistance), indicating the presence of genes that, when modulated, encode products capable of permitting or actively facilitating bypass of the requirement for estrogen in breast cell proliferation. One such locus is *BCAR1*. In this example, the cloning and sequence analysis of *BCAR1*, along with direct evidence for its role in the progression of breast cancer cells to an antiestrogen-resistant phenotype are described.

To search for transcribed sequences in this region, we retrieved cosmid clones covering approximately 80 kb of genomic DNA, and produced a cDNA library from a hybrid cell line containing the *BCAR*1 locus. Using exon-trapping and screening of cDNA libraries, transcribed regions were identified. Northern analysis showed that retrovirus integration had enhanced the transcription of a 3 kb mRNA (Figure 1). Analysis of multiple tissue northern blots (Clontech) revealed *BCAR*1 expression in most cell types. The complete cDNA has been assembled from various partial cDNA clones and was sequenced [SEQ ID NO: 1]. In addition, sequences from the exons and flanking regions were also established from the cosmid clones. Flanking sequences are shown in Figure 17 [SEQ ID NO: 7-19]; note that where uncertainty exists, bases have been underlined. Gaps in intronic sequences have been indicated by estimates of their respective lengths in parentheses. Comparison of the *BCAR*1 DNA and protein [SEQ ID NO: 2] sequences with those in the available databases revealed high homology to the rat and murine $p130^{Cas}$ protein (Sakai et al., 1994, EMBO J., 13: 3748-3756; Polte and Hanks, 1995, Proc. Natl. Acad. Sci. U.S.A., 92: 10678-10682). The overall homology indicates that the *BCAR*1 gene is the human homologue of the rat and murine $p130^{Cas}$ (Figure 2). In addition, *BCAR*1 displays homology with the human *HEF*1

and mouse *SIN* gene (Law et al., 1996, Mol. Cell. Biol., 16: 3327-3337; Alexandropoulos and Baltimore, 1996, Genes Dev., 10: 1341-1355). Homology is also observed with other protein carrying a Sarc Homology 3 (SH3) domain, which is involved in interaction with proline-rich target proteins. The strongest homology is found with the SH3 domain of Grb2, an adaptor protein involved in many signalling cascades. The central part of the protein contains many tyrosine residues in a favorable context for phosphorylation by various Src-family protein kinases. These features strongly implicate the human BCAR1 protein in signal transduction by mediating interaction between various proteins.

The full length cDNA [SEQ ID NO: 1] was introduced in the LXSN expression vector (Miller and Rosman, 1989, BioTechniques, 7: 980-990) under control of the viral LTR and transfected into the ZR-75-1 cells using Lipofectin (Gibco-BRL) or Ca-phosphate co-precipitation. Transfectants were selected for G418 resistance in estradiol-supplemented medium and characterized for protein production on western blots using a monoclonal antibody directed against rat $p130^{Cas}$ (Transduction laboratories, Catalog nr. P27820). Cell lines were subsequently seeded in medium containing 4-hydroxy tamoxifen and assayed for their proliferation capacity. Empty vector-transfected cell lines (2A-4 and 2A-5) were unable to grow under these conditions, whereas *BCAR*1 transfectants (for example, 4A-12) exhibiting *BCAR*1 protein expression showed comparable growth kinetics as a hybrid cell line (D4E6) carrying the complete *BCAR*1 locus (Figure 3; for details of somatic cell hybrid production, see Example 2, below). These results demonstrate that expression of the *BCAR*1 cDNA is sufficient to allow these breast cancer cells to proliferate in the presence of the antiestrogen hydroxy-tamoxifen. Similarly, we found that proliferation was neither affected by a pure antiestrogen (ICI 182,780) nor required the presence of an antiestrogen.

The *BCAR*1 locus was mapped to the long arm of chromosome 16 (q22-23) by in situ hybridization and partial sequence analysis revealed the presence of the chymotrypsin gene in close proximity (within 10 kb from the end of the *BCAR*1 gene). In breast cancer, under-representation or allelic loss of the long arm of chromosome 16 is frequently observed. No involvement of *BCAR*1 in these processes has been established so far.

EXAMPLE 2

In this example, a novel integration locus (*BCAR*2) from an antiestrogen-resistant cell line which can confer tamoxifen resistance to the parental, estrogen-dependent human breast cancer cells following cell fusion-mediated locus transfer is described.

Somatic cell fusion.

Cell fusion experiments were performed to distinguish loci actually involved in development of tamoxifen resistance. Donor cells were selected from our panel of OH-Tam-resistant cell lines obtained after retroviral insertional mutagenesis. The cell line VIII24, which carries two integrated retroviruses mapping outside the previously identified *BCAR*1 locus (Dorssers et al., 1993, supra) and recognizing two distinct bands on Southern blots (Figure 4), were lethally irradiated and fused *in situ* to hygromycin-B-resistant derivatives of ZR-75-1 cells.

The recipient hygromycin B-resistant variants of ZR-75-1 human breast cancer cells were obtained by transfection of a PGK-HygB^r expression construct (Te Riele et al., 1990, Nature, 348: 649-651) using cationic liposomes (Lipofectin Reagent, GIBCO-BRL, Life Technologies Ltd, Paisley, UK) as described. Stable transfectants were selected with 60 µg/ml of Hygromycin B (Boehringer Mannheim, FRG) in RPMI 1640 medium containing 10% heat-inactivated bovine calf serum (R/BCS) and supplemented with 1 nM of estradiol (Van Agthoven et al., 1992, supra). Clones which had retained complete dependence for estrogen (ZH2D2 and ZH3D7) were used as recipients in the somatic cell fusion experiments. Donor cells were either pooled colonies of ZR-75-1 cells infected with a defective LN virus (control cells) or the antiestrogen-resistant cell line VIII24 (Dorssers et al., 1993, supra).

Somatic cell fusion was performed essentially as described by Eijdems et al. (1992, Proc. Natl. Acad. Sci. U.S.A., 89: 3498-3502). Approximately 6 x10^6 donor cells, which were γ-irradiated with approx 40 Gy (Gammacel 1000, Cs137 Source) and 3 x 10^6 recipient cells were plated in 25 cm^2 flasks in R/BCS medium with estradiol. After strong adherence to the flasks in 36-48 h, cells were washed three times in RPMI medium without serum and incubated with 1 ml polyethylene glycol (PEG) 1500 (Boehringer Mannheim) at 30-34°C for 1 minute on a stretching table (Medite, Burgdorf, FRG). The PEG solution was diluted with 1, 2 and 4 ml RPMI medium after 1, 2 and 4 minutes, respectively. Finally, cells were washed three times with RPMI medium and incubated for two days at 37°C in R/BCS medium with estradiol. Selection with 1 mg/ml G418 (Geneticin, GIBCO-BRL) in R/BCS medium plus estradiol was performed for one week followed by one week of selection with 50 µg/ml Hygromycin B. After an additional week of selection with G418, colonies were picked and expanded to cell lines in R/BCS medium containing estradiol and G418. Immediate simultaneous selection with G418 (1 mg/ml) and Hygromycin B (40 µg/ml) after

fusion resulted in loss of up to half of the colonies. Double selection with 0.66 mg/ml of G418 and 20 μg/ml of Hygromycin B has been applied after the third week.

This fusion procedure generated low numbers of hybrid colonies (on average 1-2 colonies per 3 $x10^6$ recipient cells). From cell line VIII24, initially 7 colonies were picked and established (E series). These hybrids carried a single integration locus represented by either the 14kb *Hin*dIII restriction fragment (five cell hybrids) or the 5kb *Hin*dIII fragment (Figure 4). Additional fusion experiments were performed to generate further independent hybrids from the VIII24 cell line using ZH3D7 recipient cells. From 22 somatic cell hybrids, seven carried the locus represented by the 14kb *Hin*dIII fragment, 14 carried the other locus and one (E8A) contained both retroviral integration loci. Two non-related hybrids (B series), generated by fusion of an irradiated pool of LN-virus infected ZR-75-1 cells, showed distinct restriction fragments hybridizing to the NeoR probe (Figure 4).

Characterization of somatic cell hybrids.

Development of spontaneous antiestrogen resistance in ZR-75-1 cells is extremely rare ($\leq 10^{-8}$) and is significantly increased as a result of random retrovirus integration or random alteration of gene expression (Dorssers et al., 1993, supra; Van Agthoven et al., 1994, supra). Somatic cell hybrids were generated using optimal culture conditions in the presence of estradiol, since colony formation under OH-Tam selection is slow and long periods of time to are required to establish stable cell lines. Specifically, donor and recipient cells and somatic cell hybrids were plated at 0.5-1.5$x10^6$ cells per 25 cm^2 flasks and cultured in R/BCS supplemented with 1 μM 4-hydroxytamoxifen (OH-Tam, provided by ICI Farma Ridderkerk, NL), which was changed twice a week. Biological response to antiestrogen challenge was monitored by microscopic inspection. Proliferation potential of the hybrid cells in OH-Tam-containing medium was scored by microscopic inspection of anonymously labeled flasks by three investigators, DNA quantitation (Hinegardner, 1971, Anal. Biochem., 39: 197-201) and automated counting of the cells using a Technicon H1 system. In particular, cultures were evaluated for morphological properties including the presence of mitoses and increase in cell numbers. The first set of somatic cell hybrids obtained from the VIII24 cell line comprised two cell clones (E7E0 and E7E5) with a morphology (i.e. presence of mitoses and strong increase in covered area) comparable to the donor cells under OH-Tam selection (Figure 5A). The five remaining cell hybrids (E2C3, E2C5, E2F9, E5G5 and E6E8) exhibited a poor morphology (i.e. lacking mitoses and without substantial increase in cell

numbers) comparable with OH-Tam-arrested ZR-75-1 and hygromycin B-resistant recipient cells (Figure 5B).

To analyse which locus was retained in the different cell hybrids, genomic DNA was prepared, digested with informative restriction enzymes, blotted and hybridized with a Neo^R probe radiolabelled with α-^{32}P-dATP (ICN Pharmaceuticals Inc. Costa Mesa, CA) as described previously (Dorssers et al., 1993, supra). Evaluation of the proliferative capacity in the presence of OH-Tam by cell counting revealed that hybrids with the integration locus represented by the 14kb *Hin*dIII restriction fragment lacked increase of cell numbers after day 7 comparable with the recipient cell line ZH3D7 (Figure 6). In contrast, two hybrids with the other integration locus represented by the 5kb *Hin*dIII restriction fragment (E7E0 and E7E5), demonstrated slow but continuous increase in cell numbers in the presence of OH-Tam. The latter two hybrids exhibiting antiestrogen resistance were obtained from the same flask and thus may have arisen from a common fused cell.

To further investigate the relation between the integration loci in the VIII24 cells and the resistant phenotype, twenty-two additional cell hybrids were produced and analysed for antiestrogen resistance by microscopy and DNA measurement (Figure 7). The ZH3D7 cells exhibited moderate DNA synthesis during the first week (probably resulting from the delayed inhibitory effects of OH-Tam on cell proliferation), but failed to proliferate after eight days in these cultures. All 15 somatic cell hybrids with the integration locus represented by the 5kb *Hin*dIII fragment showed both a viable morphology and continuous DNA synthesis under OH-Tam (Figure 7, solid lines). Hybrid E10B displayed slow growth in the presence of OH-Tam, but also in estradiol-supplemented culture (not shown). Of the seven hybrids carrying only the integration locus represented by the 14kb *Hin*dIII restriction fragment (Figure 7, dashed lines), only two (E9C and E12B) exhibited both a viable morphology and cell proliferation in the presence of OH-Tam. The other hybrids exhibited a poor morphology and DNA synthesis was blocked after eight days.

In order to verify that the proliferation capacity of the resistant cell hybrids was sustained after the second week of selection, cells were collected, reseeded and cultured for another two weeks under OH-Tam. Most cell hybrids with the integration locus represented by the 5kb *Hin*dIII fragment showed net cell production (Figure 8, hatched bars) and continued to display a viable cell morphology during this period. Hybrids with this integration locus exhibited a proliferative capacity mostly comparable to the donor cell line.

This result indicates that the transferred locus had a dominant and immediate effect on the proliferation machinery of the majority of the cells and that these cells were actually antiestrogen resistant. In contrast, five out of seven cell hybrids with the other locus exhibited cell loss in this culture period (Figure 8, open bars). The results of the complete analyses (microscopical examination and assessment of cell proliferation) of 29 somatic cell hybrids derived from the VIII24 cells (E-series) indicate that the integration locus characterized by the 5kb *Hin*dIII fragment is associated with the antiestrogen-resistant phenotype ($p<0.001$) and thus represents the second Breast Cancer Antiestrogen Resistance locus (*BCAR2*).

Identification of the *BCAR2* locus as a common integration site.

The association of antiestrogen resistance and uptake of the *BCAR2* integration locus represented by the smaller *Hin*dIII fragment in the E-series hybrids prompted us to further investigate this region. Pulse field gel analysis using the NeoR probe indicates that the locus is not in close proximity of the *BCAR1* common integration locus. Furthermore, BCAR2 probes fail to hybridize with cosmid clones derived from the *BCAR1* region. These results indicate that a gene other than *BCAR1* is responsible for the development of antiestrogen resistance in VIII24 cells. To generate a genomic probe specific for *BCAR2*, a genomic fragment flanking the viral integration in a somatic cell hybrid (E7E5, which contains only the integration site characterized by the 5kb *Hin*dIII fragment) was generated using the inverse PCR procedure. Genomic DNA was digested with various sets of restriction enzymes generating compatible ends, religated at diluted concentration and subjected to amplification with outward-directed nested primers defined in the viral genome (Dorssers et al., 1993, supra). Product obtained from *Eae*I-digested genomic DNA, was cloned using a T-tailed vector (pCRII, Invitrogen Corp. San Diego, CA) and analysed. A 130bp fragment of genomic DNA flanking the 3' viral LTR was obtained and used to screen a lambda phage library containing human genomic DNA (HL1006d, Clontech Laboratories, Inc. Palo Alto, CA). A positive phage was isolated and subcloned into Bluescript IIKS- (Stratagene, La Jolla, CA). It was found to contain a 4.5kb *Pst*I genomic fragment overlapping the integration site and lacking repeat sequences.

This probe was used to screen Southern blots of the complete panel of 80 cell lines generated by retrovirus-mediated insertional mutagenesis (Dorssers et al., 1993, supra), for additional integration events in the *BCAR2* locus. Three cell lines (VIII24, VIII2-8a and

VIII3-3b) were identified showing an additional hybridizing fragment apart from the germ line 14kb *Bgl*II fragment detected in all cell lines (Figure 9). The additional band represents the allele containing the viral genome (approx 3kb), as the same band was also identified in VIII24 and VIII3-3b DNA by hybridization with a NeoR probe (Figure 9). The cell clone VIII2-8a was obtained from the same flask as the cell fusion donor cell line VIII24. In addition, both clones contain two viral integrations represented by fragments of apparently identical size using different restriction enzymes (*Hin*dIII and *Bgl*II), strongly suggesting that they were derived from a common infected cell. The third clone (VIII3-3b) was derived from a different virus-infected flask and contains at least four copies of the viral genome (characterized by a distinct NeoR hybridization pattern, Figure 9) and thus represents an independent integration event in this locus. These conclusions were confirmed by hybridization analysis of different restriction digests of the VIII24 and VIII3-3b cell line DNA samples (not shown). These results demonstrate that within our panel of OH-Tam-resistant cell lines, the *BCAR2* locus has acquired a retroviral genome in two independent cases and thus represents a new common integration site most likely containing the responsible gene in close proximity to the virus.

Identification of a candidate transcript in the *BCAR2* locus

Hybridization of Northern blots of BCAR2 cell hybrids with available genomic probes gave a weak indication of the presence of exon sequences. This result convinced us to use these probes for cDNA selection strategies on amplified cDNA from BCAR2 cell lines. All repeat-free fragments of the genomic phage were biotinylated and hybridized with the cDNA. Selected cDNA was re-amplified and subjected to further rounds of cDNA selection. Various products were obtained after cloning, some displaying non-linearity with the genomic DNA. Two distinct exons (nucleotides 1-31 and 32-116) present in these selected cDNAs were confirmed by sequencing parts of the genomic clones in the integration region. A representative clone of the selected cDNA clones was used for Northern analysis and identified transcripts of approximately 5 and 8 kb in BCAR2 cells (E17A), but these transcripts were barely detectable in the original ZR-75-1 cells. The same transcripts were also identified in MDA-MB231 cells, MCF7 cells and on human multiple tissue Northern blots (Clontech) with the highest expression observed in testis and kidney. cDNA libraries from these sources were screened and provided two overlapping cDNAs (nucleotides 1-1922). Since no additional cDNA clones were found, we utilized RACE strategies (Clontech

Marathon-Ready testis cDNA) and cDNA selection to extend our BCAR2 gene sequences. RACE to the 5'-end indicated that the previously isolated cDNA's were derived from the extreme 5'-end of the transcript and demonstrated some alternative splicing upstream of nucleotide position 32. Extension to the ultimate 3'-end was achieved by multiple rounds of RACE and cDNA selection cycles.

Since the expression of these transcripts in all tissues appears quite low, we wished to certify that mRNA sequences containing an open reading frame were being pursued. We tested this using *in vitro* transcription-translation (ITT). The first construct was assembled from the testis and kidney cDNA clones (nucleotides 1 to 1922) and inserted behind a vector ATG codon in three different reading frames in a His-tag vector. Following transcription with T7 RNA polymerase and simultaneous translation using biotinylated lysine-tRNA complex, proteins were analysed by Western blotting. Expression constructs in three reading frames using the vector ATG codon gave rise to the same translation product (approx. 50 kDa), which did not correspond to the translation of the complete cDNA. Similarly, expression vectors without a vector ATG codon also gave rise to a similarly-sized polypeptide. This indicated that translation from the vector ATG was blocked by downstream in-frame translation stop codons and that an internal ATG was utilized. Deletion constructs in which the first 573 bp were removed indeed gave a His-tagged translation product of approximately 52 kDA from the vector ATG in a single reading frame. The ATG codon at position 570-572 of the cDNA fits the Kozak consensus and is most likely the actual start site for translation of this mRNA. The complete assembled constructs tested so far by *in vitro* transcription and translation, containing either the short version or the longest version of the cDNA, gave rise to identically-sized proteins of approximately 150kDa.

Sequence analysis of both strands is complete for the longest expression construct tested. The *BCAR2* cDNA [SEQ ID NO: 20] is 7616 bases long and contains a long open reading frame (nucleotides 570 to 4169) corresponding to a putative protein of 1200 amino acids [SEQ ID NO: 21] with a molecular weight of 132 kDa. An internal site of polyadenylation was observed at nucleotide position 4390, explaining the occurrence of smaller cDNA clones and transcripts of approximately 5 kb. Since part of the assembled cDNA clone is derived from PCR amplified cDNA (nucleotides 1923 to 7617), the presence of polymerase mistakes cannot be excluded. Variant bases observed in other sequenced RACE and cDNA-selection fragments have been summarized in Table 1. With the exception

of the change at position 2576, all other changes identified so far were present in single clones. In addition to nucleotide substitutions, splice variants have been observed. Variant cDNAs were found at the extreme 5'-end (upstream of position 32), but did not affect the encoded protein. Within the coding region, an insert of 60 bp [SEQ ID NO: 22, encoding amino acid sequence of SEQ ID NO: 23] was observed at nucleotide position 2454 of our assembled cDNA. This insert was present in cDNA from testis and from the breast cancer cells recovered with RACE and cDNA selection strategies, respectively. A set of variant cDNAs (at nucleotide position 3636), was retained from RACE experiments on testis cDNA. The predominant variant has been used for the sequence determination. Other variants contained either an insertion of 36 bp [SEQ ID NO: 24] (i.e. 12 amino acids, SEQ ID NO: 25), or upstream deletions of 120bp (up to nucleotide 3515) or 207bp (up to position 3428) corresponding to the loss of 40 or 69 amino acids, respectively. The insertion and deletion events disrupt the second C2H2-type Zn-finger domain (amino acids 1015-1037) in the protein. The frequencies of these variant mRNA in BCAR2 expressing cells and the role of these in-frame alterations on the function of the protein need to be established.

Database search for homologous proteins and protein domains reveals the presence of three C2H2-type Zn-finger motifs (amino acid positions 514-534, 1015-1037, 1088-1108). Furthermore, a region (amino acids 888-933) showing 36% amino acid identity with part of human metastasis-associated protein MTA1 (GenBank U35113) was found. Additional homology in adjacent amino acids were found with proteins of various species: C. elegans (Z77664), X. laevis (AF015454) and rat (A54766). No specific function has as yet been assigned to this particular domain.

Thus, a novel differentially-expressed cDNA has been isolated from the BCAR2 integration locus, which may be responsible for anti-estrogen resistance in breast cancer cells.

Table 1

Position	Alteration	Amino Acid change
44	C → G	
61	G → A	

315	G → T	
344	G → A	
368	A → T	
369	G → A	
534	T → C	
984/85	GT → AC	Val → Thr
1396	A → C	Gln → Pro
1683	G → T	Gly → Cys
1805	T → C	No change
2200	A → G	Lys → Arg
2361	A → G	Ser → Gly
2430	T → C	Ser → Pro
2454	60 bp insert	20 amino acid insert
2470	A → G	His → Arg
2576	G → A	Cys → Tyr
2658	C → T	No change
2850	T → C	No change
2965	C → A	Ala → Asp
3164	G → A	Met → Ile
3405	G → A	Ala → Thr
3429-3635	207 bp deletion	69 amino acid deletion
3516-3635	120 bp deletion	40 amino acid deletion
3636	36 bp insertion	12 amino acid insertion
4176	C → G	
4340	A → T	
4380/81/82	TTG → ATG	

EXAMPLE 3

Common site of integration *BCAR*3.

To identify genes involved in antiestrogen resistance, cell clone X-3-6 was selected from the panel of 80 tamoxifen-resistant cell clones from which cells bearing *BCAR1* and *BCAR2* were isolated in Examples 1 and 2. Cell clone X-3-6 contains only one integrated provirus which is expected to induce the resistant phenotype and does not belong to the clones which contain a viral integration in the *BCAR*1 or *BCAR*2 loci. To screen for additional clones with an insertion in this region an integration site specific probe was created (Figure 10A). First, chromosomal DNA of clone X-3-6, was isolated using NaCl extraction procedures described by (Miller et al., 1988, Nucleic Acids Res., 16: 1215), digested with a panel of restriction enzymes and analysed by Southern blotting techniques. Hybridization using a retroviral-integration-specific NeoR probe that had been labelled with α-^{32}P dATP (ICN Pharmaceuticals Inc. CA) *via* random priming showed that a combination of BamHI and BglII digest resulted in a restriction fragment of approximately 6.5 kb, convenient for isolation of a large fragment adjacent to the viral integration (Figure 10A). DNA of cell clone X-3-6 cleaved with BamHI and BglII restriction enzymes was circularized by ligation and amplified using outward directed primers located within the NeoR and viral *gag* sequences. Subsequently, this inverse PCR product was amplified with two sets of nested primers located within the LTRs (Figure 10A). This approach resulted in a 3.2 kb PCR fragment of genomic sequences adjacent to the viral integration site. Alu-repetitive sequences were removed from this fragment by subcloning which resulted in a 1.6 kb probe (Figure 10A).

Southern analysis of X-3-6 and parental ZR-75-1 DNA digested with a panel of restriction enzymes and hybridized with the integration- and a NeoR probe, confirmed the specificity of this probe for the X-3-6 integration site. Subsequently, the cell line panel was screened for the occurrence of a common site of integration. Southern blots of the 80 antiestrogen resistant cell lines containing DNA, digested either with the restriction enzymes BamHI, BglII or HindIII, were hybridized with the integration specific probe of clone X-3-6. Figure 10B shows that this probe identified a 15 kb HindIII germ line restriction fragment in ZR-75-1 cells and in the derived integration cell clones. In the X-3-6 cell clone an additional fragment was observed, resulting from integration of the LN retrovirus (approximately 3 kb)

in the other allele (Figure 10B). This analysis revealed 5 additional cell clones with a provirus integrated in this region. In total a region of approximately 25 kb surrounding the X-3-6 integration site was covered. Additional integrations further up- or downstream this region can not be excluded. Detailed mapping analysis of the 6 integration sites demonstrated that they were distinct and thus confirmed the independent origin of the clones (Figure 10A). In addition, Southern analysis and PCR mapping showed that all clones have similar 5' to 3' direction of transcription of the retrovirus. Five viral integrations have occurred in a 1 kb region and one, in clone X-1-2B, is integrated 5 kb upstream. The occurrence of 6 retroviral insertions in 6 independently arisen cell lines in a panel of 80 cell lines in a region of 5 kb is unlikely to be the result of a random process ($2.9x10^{-19}$, binomial distribution, Dorssers et al., 1993, supra). This common site of integration is thus tightly linked with antiestrogen resistance and is termed Breast Cancer Antiestrogen Resistance 3 (*BCAR*3).

Transfer of the *BCAR*3 locus by somatic cell fusion.

To determine whether the alteration in the *BCAR*3 locus, caused by retroviral mutagenesis, is a dominant or recessive event, somatic cell hybrids were generated. The acceptor cells were ZR-75-1 cells (designated ZH3D7) transfected with the expression construct PGK-Hyg B^r, which confers hygromycin B resistance. Integration clone XI-1-6A, which had been shown by Southern analysis to carry two integrated proviruses, one in the *BCAR*3 locus and an additional integration which is expected to be non-relevant for antiestrogen resistance, was selected as the donor cell line, and was lethally irradiated to prevent outgrowth that did not result from fusion with the acceptor cells. As a consequence of the retroviral insertions these donor cells are both antiestrogen- and G418-resistant; the presence of the additional proviral integration in this cell line serves as an internal control. ZH3D7 and XI-1-6A cells were fused with polyethylene glycol (PEG, Boehringer Mannheim, FRG, Germany) using a procedure for adherent cells (Eijdems et al., 1992) and according to the manufacturers recommended conditions. After fusion, the cells were allowed to recover for two days prior to a 10 days of selective growth on 1 mg/ml G418 (Geneticin, Gibco-BRL, Life Technologies Ltd, Paisley, UK) in the presence of 1 nM 17 beta-estradiol. The selection was subsequently continued with 500 µg/ml G418 and 25 µg/ml Hygromycin B (Boehringer Mannheim) until clones developed, to ensure that all viable hybrids not only had acquired, but continued to maintain at least one of the NeoR-containing

integration loci.

The 11 hybrids so obtained were characterized with a diagnostic HindIII restriction digestion to distinguish between the two viral integrations. Five hybrids contained viral integration in the *BCAR*3 locus and six hybrids carried the virus in the other integration site. Subsequently, the hybrids were tested for antiestrogen resistance. The growth performance of triplicate cultures of the cell hybrids was compared with the parental ZR-75-1, ZH3D7 and XI-1-6A cells in culture medium containing 1 μM of 4-hydroxy-tamoxifen. Antiestrogen sensitivity of cell hybrids was scored on days 7 and 10. Cultures of hybrid cells showing poor, rounded cell morphology, loose attachment to the culture flask, low numbers of mitoses and little or no increase in cell numbers (measured as the percentage of covered surface, ~20-30%) comparable with ZR-74-1 and ZH3D7 cells were scored 4-hydroxy-tamoxifen-sensitive (Fig. 11B). Hybrids which looked healthy, with flattened morphology, firmly attached to the culture flask, with readily visible numbers of mitoses and cell numbers occupying up to 90% of the surface of the culture flask, comparable with XI-1-6A cells, were scored tamoxifen-resistant (Fig. 11A). Only the 5 hybrid cell lines carrying the *BCAR*3 locus could proliferate in the presence of tamoxifen (Figure 11A), while those without it failed to multiply (Figure 11B), indicating that transfer of the *BCAR*3 integration locus confers dominant resistance to the parental ZR-75-1 cells.

Identification of coding sequences in the *BCAR*3 locus.

In five out of six resistant cell lines the integration of the retrovirus occurred in a very narrow region (Figure 10). This indicates a positive selection for this region and possibly a common mechanism of alteration of gene expression. Northern blots with RNA of the integration clones were screened with the genomic inverse PCR probe to identify possible transcribed sequences. Large transcripts were observed in the *BCAR*3 clones, which vary in size in the various cell clones and were not detected in the parental ZR-75-1 cells (Figure 12). Northern blot hybridization demonstrated that these are chimeric transcripts composed of both LTR, NeoR and *BCAR*3 sequences (data not shown). The observed differences in mRNA sizes in individual cell clones can be explained by the position of the integrated retroviruses to the *BCAR*3 sequences and read through transcripts from the viral LTR. (Figure 10 and Figure 12). More important, screening of Northern blots (Figure 12) with RNA of a panel of cell lines revealed a transcript of approximately 3.4 kb in the estrogen-independent MDA-MB-231 breast cancer cell line (Cailleau et al., 1974, J. Natl. Cancer Inst., 53: 661-

674). This indicates that this genomic integration probe contains exon sequences. This *BCAR*3 transcript appears also to be present in the integration clones and is barely detectable in the ZR-75-1 cells. Estrogens can modulate gene expression of target genes. However, *BCAR*3 is not detectably regulated in ZR-75-1 cells. Cultivation of ZR-75-1 cells in the presence and absence of estradiol and in the presence of tamoxifen did not result in upregulation of *BCAR*3 expression (Figure 12). Therefore, most likely the observed upregulation of the *BCAR*3 RNA is induced by the integrated virus.

*BCAR*3 cDNA cloning and predicted Amino Acid Sequence.

To isolate the gene corresponding to the observed transcript, a human testis cDNA library (Clontech Laboratories, Inc. Ca) was screened with the *BCAR*3 integration-site-specific probe. Plaques were purified and the cDNA inserts were recloned in pGEM4Z (Promega, Madison, WI). In the first screening round, a 1.9 kb cDNA clone was isolated (#16, Figure 13). The nucleotide sequence [SEQ ID NO: 3] was determined on both strands by dideoxy sequencing reactions using T7 DNA polymerase (Pharmacia Biotech, Sweden) and α-^{32}P dATP (ICN Pharmaceuticals Inc.). Sequence comparison of the genomic integration probe with the cDNA showed that the genomic probe contains a 133 bp exon, identical to sequences of the cDNA clone #16 (represented by nucleotides 456 to 589 of SEQ ID NO: 3) with consensus splice donor and acceptor sites. Northern blot analysis confirmed that this cDNA clone recognizes the *BCAR*3 transcripts. In successive screening efforts to obtain the full length cDNA overlapping clones were isolated (Figure 13). The assembled cDNA consists of 3004 base pairs, while the mRNA is estimated to be 3.4 kb in length, based upon its eletrophoretic mobility on Northern blots. The first ATG is not preceded by an in-frame stop codon. Therefore, a 5' RACE (rapid amplification of cDNA ends) strategy was performed to obtain the additional sequences at the 5' end of the *BCAR3* mRNA. Total RNA of MDA-MB-231 cells, which show abundant expression of *BCAR3*, was used to obtain the full coding region of *BCAR3*. Several 5' RACE clones were isolated and sequenced. It was shown that an in-frame stop codon (TGA) is present 72 bp upstream of the first ATG. The *BCAR3* cDNA obtained is 3042 bp in total and contains the complete coding region. Three ATGs are present in the first 174 base pairs of the cDNA. Analysis of the surrounding sequences of the 3 putative start sites indicates that the first ATG codon at position 99 fits the Kozak consensus sequence (Kozak, 1991). The open reading frame is flanked by an in-frame TGA translation termination codon at position 2574, followed by a 448-nucleotide 3'

untranslated region and a poly(A) tail of 19 nucleotides. Assuming that translation starts at the first ATG at position 99, *BCAR*3 has a single open reading frame that encodes a protein of 825 amino acids with a predicted molecular mass of 92 kDa [SEQ ID NO: 4]. Comparison of the sequence of *BCAR*3 with the available protein and nucleotide data bases revealed that *BCAR*3 is a newly identified gene with a single stretch of amino acids (numbers 154-253 of SEQ ID NO: 4) with strong homology with Src homology 2 (SH2) domains of other proteins. A profile scan utilizing the Prosite profile data base detected no further putative protein domains. However, homologous sequences to part of the yeast cell division cycle protein CDC48 were observed by using the BLASTP algorithm (amino acids 699-812; SEQ ID NO: 4).

mRNA expression of *BCAR*3

We next evaluated the expression of *BCAR*3 mRNA in various normal tissues. For this purpose commercially- available Northern blots were hybridized with the 1.9 kb cDNA probe clone #16. *BCAR*3 mRNA is widely expressed and abundant transcripts were observed in heart, placenta, skeletal muscle, testis, ovary and fetal kidney. In skeletal muscle and heart an additional 6 kb mRNA is present (Figure 14). The nature of this larger transcript is at present unclear but may be explained by alternative splicing and/or alternative promoter usage. In addition, Northern analysis was performed on RNA isolated from non-malignant breast tissues. No expression of *BCAR3* was detected in those samples ($n = 6$). However, the number of luminal epithelial cells in normal breast tissue is small in comparison to the number of stromal cells; therefore, expression of *BCAR3* by glandular epithelial cells cannot be excluded. A panel of cell lines originating from breast, ovarian and endometrial cancer was screened by Northern analysis for the expression of *BCAR3*. These results were compared with ER and EGF receptor protein expression (Table 2).

Table 2. Expression of *BCAR3* mRNA was compared with expression of ER and EGF receptor protein

Cell line	BCAR3	ER	EGFR
Breast			
ZR-75-1	-	+ (100)	- (0)
MCF-7	-	+ (300)	- (20)
T-47D	-	+ (100)	+ (600)
BT-474	-	+ (23)	nd

BT-20	++	- (<10)	nd
EVSA-T	-	- (<10)	+ (67)
HS-578-T	+	- (<10)	nd
MDA-MB-134	-	- (<10)	nd
MDA-MB-231	+++	- (<10)	+ (>1600)
SK-BR-3	-	- (<10)	+ (560)
HBL-100	+	- (<10)	nd
RC-6	-	- (<10)	nd
Ovary			
2780	-	- (<10)	+ (220)
2774	++	- (<10)	+ (*)
HOC-7	+	- (<10)	+ (*)
KB3.1	+	- (<10)	nd
OVCAR-3	+	- (<10)	+ (1200)
SK-OV-3	+	- (<10)	+ (>1600)
SK-OV-6	+	- (<10)	+ (>1600)
Endometrium			
ECC-1	-	+ (82)	+ (84)
AN-3-CA	+	- (<10)	+ (1550)
HEC-1-A	++	- (<10)	+ (>1600)
HEC-1-B	+	- (<10)	+ (>1600)
KLE	-	- (<10)	+ (530)
SCRC	++	- (<10)	+ (>1600)
RL 95-2	++	- (<10)	+ (>1600)

Receptor concentrations determined with biochemical assays are expressed as fmol/mg protein. Expression of ER > 0 fmol/mg and EGF receptor > 50 fmol/mg was scored positive (+). ND not done. (*) Expression determined by Northern analysis. Expression levels of *BCAR* varied between - , no expression detected after 7 days exposure, and +++, high expression detected after overnight exposure on film.

In these cell lines only the 3.4 kb mRNA was detected. In breast cancer cell lines tested (ZR-75-1, MCF7, T47D and BT-474, estrogen receptor-positive) and (EVSA-T, SK-BR-3 and MDA-MB-134, estrogen receptor-negative) no expression of *BCAR3* was detected. In the ER-negative breast cancer cells BT-20, HS-578 and MDA-MB-231, abundant expression of *BCAR3* was observed. In two ER-negative immortalized mammary epithelial cell lines, RC-6 and HBL-100, only the latter showed expression. In ovarian cell lines (SKOV3, SKOV6, OVCAR3, 2774, HOC7, KB3.1 and 2780, all estrogen receptor-negative) high expression of *BCAR*3 was observed in all but 2780. In the endometrial cell lines tested (SCRC, AN-3-CA, HEC1A, HEC1B, RL 95-2 and KLE, estrogen receptor-negative and ECC1, estrogen receptor-positive) high mRNA levels were observed in all except for ECC1 and KLE. The integrity of the mRNA on Northern blots was examined by hybridization to a GAPDH or actin probe. Correlation of ER and EGF receptor expression with expression of *BCAR3*

showed an inverse relationship between expression of *BCAR3* and ER (Mc Nemar test, $P = 0.025$).

In Vitro transcription translation of *BCAR3*

Figure 10 and Figure 13 show that all proviral integrations had occurred in an intron in the 5' region of the *BCAR3* gene, most likely resulting in a shorter mRNA. Northern blot analysis with a specific probe for the 5' part of the gene (represented by nucleotides 38-420; SEQ ID NO: 3) demonstrated that these sequences were not present in the shortened *BCAR3* mRNA of the integration clones, but were present in the MDA-MB-231 *BCAR3* transcripts (not shown). The shortened *BCAR3* gene in the antiestrogen-resistant clones may encode a protein of 699 amino acids with a predicted mass of 78 kDa, assuming that the ATG codon at position 477 of SEQ ID NO: 3 is the initiator methionine. Both a near full-length *BCAR3* (nucleotides 38-2989) and a shortened construct (represented by nucleotides 421-2989) resembling the presumed situation in the *BCAR3* clones were subcloned into a vector which allowed the expressed protein to start with the authentic ATG codon. The plasmid was transcribed from the T7 promoter and translated in rabbit reticulocyte lysate by using the TNT Coupled Reticulocyte Lysate Systems (Promega). After SDS/PAGE, recombinant proteins were electrotransferred to nitrocellulose membranes and proteins were detected with Non-Radioactive Translation Detection System (Promega) and chemiluminicense (Amersham International, Bucks, UK) by exposing to X-ray film (Eastman Kodak Company, NY).

The protein produced by *in vitro*-transcription and translation of full-length *BCAR3* migrated as a single band by SDS-page at approximately 90 kDa (Figure 15), while that produced by the shortened construct (represented by nucleotides 421-2989) showed a single band of approximately 74 kDa (Figure 15).

Ectopic expression of *BCAR3* induces estrogen independence

Stable transfectants with the *BCAR3* gene were established to provide conclusive evidence that *BCAR3* is the gene responsible for antiestrogen resistance in this locus. For this purpose two expression constructs were made containing a *BCAR3* cDNA. A near full-length *BCAR3* cDNA (#32, nucleotides 38-2989) was cloned into the pLXSN and pLNCX expression vectors (Miller and Rosman, 1989, supra) in which transcription is driven by the LTR promoter and in the latter by a cytomegalovirus promoter. Expression constructs and control vectors without inserts were transfected into ZR-75-1 cells using Lipofectin Reagent

(Life Technologies). Cells (2 x 10^5) were seeded in 25 cm^2 flasks (Costar, Cambridge, MA) in medium containing 17 beta-estradiol. After two days the cells were washed twice with serum free Optimem medium (Life technologies), then placed in 3 ml Optimem medium with 30 µl Lipofectin and incubated for 1 hour at 37°C, after which time 2-5 µg linearized plasmid DNA was added. Following 5 hour incubation, the DNA containing medium was replaced for RPMI medium, containing 10% BCS and 1 nmol/L 17 beta-estradiol. Subsequently, after two days, selection with G418 was started. Individual transfectants were isolated and propagated in medium with estradiol and G418.

These transfectants, designated ZR/BCAR3 cells, were expanded and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated bovine calf serum (BCS) and 1 nmol/L 17 beta-estradiol. We determined the proliferative capacity of the *BCAR3* transfectants in the presence of the antiestrogen 4-hydroxy-tamoxifen. To accomplish this, cells cultivated in the presence of 17 beta-estradiol were harvested by treatment with trypsin-EDTA. Single cells (0.7 x 10^6) were plated in 25 cm^2 plastic culture flasks in triplicate. The experimental medium containing 10% BCS and 1 µM 4-hydroxy-tamoxifen was changed two times per week. After 11 days, the cultures were harvested by trypsinization and counted. Subsequently 1.5 x 10^6 cells were replated to determine secondary growth rate. Figure 16 charts the increase in cell numbers over an 11-day culture period of parental ZR-75-1 cells, two vector controls and eight independently-derived transfectants. Parental ZR-75-1 cells, which are fully dependent on estradiol for proliferation, give rise to no more than one generation in the presence of tamoxifen (Figure 16A). As expected, similar results were obtained with the LNCX and LXSN vector controls. In contrast, the ZR/BCAR3 cells had acquired the ability to proliferate in the presence of tamoxifen or the pure antiestrogen ICI 182,780. In addition, the proliferative capacity of the transfectants is similar in the presence or absence of antiestrogens and thus not dependent on antiestrogens. Subcultivation after the 11 day culture period of ZR-75-1 cells and transfectants with the vector alone, in the presence of tamoxifen, resulted in rapid decline of these cultures. Transfectants C5, C9, C10 with *BCAR3* under control of the CMV promoter were successfully subcultured and became expanding cultures. Cell clone L24 of the *BCAR3* transfectants with the LTR promoter only developed as a stable estrogen-independent cell line. The reason for these variations are at present not clear, but may be attributed to differences in BCAR3 protein levels. In total, 40 transfectants were tested and 25 (62.5 %) showed objective growth in the presence of

tamoxifen. Similar results were obtained with the antiestrogen ICI 182,780, a pure antagonist of estrogen-stimulated proliferation. None of the 10 vector control cell lines and the parental ZR-75-1 cells, which were repeatedly tested, were able to grow in the presence of tamoxifen or ICI 182,780.

Additional experiments were performed to establish whether *BCAR3* can induce antiestrogen resistance in the ER-positive MCF-7 breast cancer cell line. MCF-7 cells were transfected with *BCAR3* under control of the CMV promoter (MCF7/BCAR3) or with the LNCX vector without the insert (MCF7/LNCX). Both pools of stably-transfected cells and individually-established clones were obtained in medium containing 10% fetal calf serum (FCS) and G418. In total, twenty MCF7/BCAR3 clones and eight MCF7/LNCX vector clones were isolated and tested for proliferation ability in the presence of the pure antiestrogen ICI 182,780. Transformant cell lines and parental control cells (MCF-7) were plated in medium supplemented with 10% FCS and 100 nM of ICI 182,780 in triplicate at an initial density of 0.25×10^6 cells *per* 25 cm^2 flask. Fourteen out of 20 MCF7/BCAR3 clones (70%) showed objective growth as compared with the MCF7/LNCX clones. Similar results were obtained with cultures in the presence of 4-hydroxy-tamoxifen. Growth curves of the pools of transfectants and the parental MCF7 cells in the presence of antiestrogen are presented in Figure 16B, in which cumulative cell numbers observed over a 50 day culture period are shown, and demonstrate the requirement for *BCAR3* for antiestrogen-resistant proliferation. Cells were counted on the indicated days and replated at the initial cell density.

These results, in which transfection of the near full-length cDNA to the parental ZR-75-1 cells resulted in the bypass of hormone dependence and immediate antiestrogen resistance, suggest that upregulation of *BCAR3* stimulates an alternative, hormone-independent growth pathway, in either the presence or absence of tamoxifen. In established cell lines derived from breast, ovarian and endometrial cancers, an inverse relationship in expression of *BCAR3* and the estrogen receptor was observed, as was a strong correlation between expression of *BCAR3* and the EGF receptor (not shown). The strongest predictor for response to tamoxifen is the expression of a functional estrogen receptor in the primary tumor (Foekens et al., 1994, Brit. J. Cancer, 70: 1217-1223; Spyratos et al., 1994, Brit. J. Cancer, 69: 394-397; Johnston et al., 1995, Cancer Res., 55: 3331-3338). Substantial clinical evidence also exists for the involvement of the EGF receptor in the malignant progression of breast cancer, in that expression of EGF receptor has been associated with lack of response to

endocrine therapy (Nicholson et al., 1988, Brit. J. Cancer, 58: 810-814; Nicholson et al., 1989, The Lancet, 1(8631): 182-185; Klijn et al., 1992, Endocr. Rev., 13: 3-17). Hence, *BCAR*3 may be an as yet unidentified component of the EGF receptor signalling pathway, although in the *BCAR*3 transfectants no expression of EGF receptors was detected. Overexpression of *BCAR*3 appears sufficient to bypass the requirement for the EGF receptor itself. BCAR3 may be a downstream component of the EGF receptor pathway, or activate other signalling pathways, resulting in hormone independence.

EXAMPLE 4

In the above Examples, it was established that enhanced transcription of the *BCAR1* gene, brought about by retroviral insertion, accompanied antiestrogen-resistance and that expression of either a *BCAR1* or a *BCAR3* transgene in cultured cells was sufficient to convert ZR-75-1 cells to an antiestrogen-resistant phenotype. Somatic cell hybrids that had acquired a single copy of either of the retrovirally-mutated *BCAR1, BCAR2* or *BCAR3* loci achieved antiestrogen resistance; further, in Example 3, it was shown that the level of *BCAR3* transcript is enhanced in antiestrogen-resistant cell lines bearing a retroviral insertion at that locus, as well as in estrogen-independent MDA-MB-231 breast cancer cells. These results indicate that the conversion of breast tumor cells to an antiestrogen-resistant state by the several *BCAR* genes occurs by a dominant mechanism, one which involves upregulation of any one of these genes.

It is intended that the methods of the invention, particularly measurement of the expression levels of the BCAR1, BCAR2 and BCAR3 proteins in tumor cells, be applied to clinical situations in which one is presented with tumor biopsy tissue requiring evaluation with regard to its state of estrogen-dependence. In order to render the results of such measurements useful toward the assessment of a patient's prognosis for treatment and recovery, it is necessary to make an initial determination of the "normal" level of expression of those genes or of any other marker to be similarly used (see Example 5, below). Significant deviations from such a value is indicative of an abnormal condition.

The difference between the normal versus the abnormal state is typically defined through the analysis of a large series of patient biopsies, in which the resulting data is correlated with a clinical marker (for example response to therapy or survival). The general procedures are based on isotonic regression analysis (Barlow et al., Statistical interference under order restrictions, 1972, John Wiley & Sons, Inc., London; also see Foekens et al.,

1994, J. Clin. Oncol., 12: 1648-1658) or on a maximal likelihood determination of cutoff value (Tandon et al., 1990, N. Engl. J. Med., 322: 297-302). In both cases, a defined assay is used to determine the level expression of the (molecular) marker and subsequently the cutoff point giving the largest difference in clinical effect is calculated. Either method offers a means of calculating normal values of expression level of the several *BCAR* genes when they are used as diagnostic tools.

Preliminary quantitation of the relative levels of *BCAR1* and *BCAR3* expression in antiestrogen-resistant cells has yielded initial baseline values for these two genes. The level of BCAR1 protein was compared in the parental ZR-75-1 cells and an antiestrogen-resistant hybrid carrying the activated locus. On Western blot, the level of BCAR1 protein was approximately 7-fold higher in the antiestrogen-resistant variant compared to the ZR-75-1 cells. In addition, a small series of normal breast tissue biopsies was examined, and no detectable expression of the BCAR1 protein was found on Western blots; therefore, the level of expression in this tissue is much lower than in ZR-75-1 cells.

Scanning of the autoradiograph in Figure 12 indicates that the level of *BCAR3* mRNA in the estrogen-independent MDA-MB-231 cells is more than 30-fold higher than in ZR-75-1 cells. The expression levels in the resistant cell lines with a virus integrated in the *BCAR3* locus are not relevant for this analysis. Screening of Northern blots with RNA from normal breast tissue (a limited series) indicates that the level of BCAR3 in this tissue does not exceed the level in ZR-75-1 cells.

Either Northern or Western analysis can be used on samples derived from tumor tissue according to the methods of the invention, as described in the above Examples for samples prepared from tissue culture cells; however, where the goal is the detection of what may be a small population of antiestrogen-resistant cells, or even a single such cell, in a tumor, it is preferred that *in situ* hybridization of *BCAR* probes to mRNA or immunocytochemical analysis using antibodies directed at the BCAR proteins be performed on fixed sections of tumor biopsy tissue. Methods for these histological procedures are currently in clinical use, and should be known to any worker skilled in the art (see Example 6, below).

An observed deviation in the level of *BCAR* gene or protein expression of at least 5 fold, preferably 15-fold, 30-fold, 100-fold or even 500-fold, relative to normal in a cell is indicative of malignant growth, endocrine independence or, more specifically, estrogen

independence.

EXAMPLE 5

According to the methods of the invention, one can assess cell malignancy, endocrine independence or, particularly, estrogen independence by measuring the ratio of expression levels of two genes relative to each other in a normal cell, performing the same measurement in a sample of tumor cells and comparing the two ratios. A shift in the ratio is indicative of abnormal expression of one gene or the other. A diagnostic panel of genes can be assembled to comprise genes selected such that their levels of expression change when a normal cell becomes malignant, when an endocrine-dependent cell becomes endocrine-independent or when an estrogen-dependent cell makes the transition to estrogen independence. We have already demonstrated, in the above Examples, that three such genes are *BCAR1*, *BCAR2* and *BCAR3*. In cell lines tested to date, the amount of BCAR3 protein is inversely related to that of ER. Cells that express ER, PR or PS2 at normal levels have very low levels of BCAR3. Conversely, BCAR3 expression is high in cells lacking ER, PR and PS2 (data not shown).

In primary tumors, expression of the epidermal growth factor receptor (EGFR) and amplification of *HER2/NEU* is known to predict a response failure to tamoxifen therapy (Nicholson et al., 1989, supra; Berns et al., 1995, Gene, 159: 11-18). Transduction of estrogen-dependent ZR-75-1 cells with an expression vector carrying the epidermal growth factor (EGF) receptor resulted in proliferation independent of estradiol (Van Agthoven et al., 1992). Ectopic expression of specific genes (activated *RAS*, insulin-like growth factor II, *EGFR*, *HER2/NEU*, *FGF*-4, *TGFβ*1 and Cyclin D1) was shown to change the hormone-dependent phenotype (Musgrove et al., 1994, Proc. Natl. Acad. Sci. U.S.A., 91: 8022-8026). Furthermore, in different experiments we showed that random changes in the methylation pattern of the DNA, induced by 5-azacytidine, resulted in a high frequency of antiestrogen-resistant cell clones (Van Agthoven et al., 1994, supra). These results indicate that several mechanisms by which to escape hormone dependence exist. Any of these genes is suitable for inclusion in a diagnostic panel along with one or more of *ER*, *PR* and *PS2* to be assayed in conjunction with one or more of *BCAR1*, *BCAR2* and *BCAR3* for clinical assessment of hormone dependence in breast- and other estrogen-dependent cancers. Suggested panels, which are not meant to be limiting, are as follows:

a) One or more of BCAR1, BCAR2 and BCAR3 measured against one or more of ER, PR and PS2.

b) One or more of BCAR1, BCAR2 and BCAR3 measured against one or both of EGFR and HER2/NEU.

c) One or more of BCAR1, BCAR2 and BCAR3 measured against one or more of activated RAS, insulin-like growth factor II, FGF-4, TGFß1 and Cyclin D1.

Statistical analysis to determine normal levels of expression of these genes or proteins is performed as in Example 4; from the values generated by that analysis, one can calculate the normal ratios of expression of the various genes or proteins relative to one another. Statistically-significant deviation from these ratios in a nucleic acid or protein sample derived from tumor cells (or in tumor cells themselves, if *in situ* techniques are to be used in place of Northern or Western analysis, which is suggested, in light of points raised in Example 4 with regard to cell number; see Example 6) relative to those observed in normal cells is indicative of antiestrogen-independent tumor cell growth. An observed deviation in the ratio of expression levels of one or more *BCAR* gene or protein and a second gene or protein of the panel of at least 2-fold, preferably 10-fold, 20-fold, 100-fold, 200-fold, 1,000-fold or even 10,000-fold, relative to that observed in a normal cell is indicative of malignant growth, endocrine independence or, more specifically, estrogen independence.

EXAMPLE 6

It is desirable to be able to detect a small number of malignant cells or cells that are endocrine-independent or, more specifically, estrogen-independent, from within the context of a larger population of tumor cells. This can be achieved by direct visualization of one or more of the BCAR1, BCAR2 and BCAR3 proteins on immunostained sections of tumor tissue, whereby the protein products of a single cell are not diluted out by those deriving from other cells of the sample, and subtle changes in the expression level of a gene can be observed. Note that such an argument also holds for mRNA detection in *in situ* hybridization procedures. This method illustrated in this Example is designed to provide for examination either of BCAR protein expression levels (as in Example 4) or the ratio of levels of expression between members of a panel of proteins (as in Example 5).

Tumor biopsy material can be fixed using conventional materials; either formalin or a multi-component fixative, such as FAAG (85 % ethanol, 4% formaldehyde, 5% acetic acid, 1% EM grade glutaraldehyde) are adequate for this procedure. Tissue should be fixed at 4°C, either on a sample roller or a rocking platform, for 12 to 48 hours in order to allow fixative to reach the center of the sample. Prior to embedding, samples must be purged of

fixative and dehydrated; this is accomplished through a series of two-to-ten minute washes in increasingly high concentrations of ethanol, beginning at 60%- and ending with two washes in 95%- and another two in 100% ethanol, followed two ten-minute washes in xylene. Samples can be embedded in a variety of sectioning supports; paraffin, plastic polymers or a mixed paraffin/polymer medium (e.g. Paraplast®Plus Tissue Embedding Medium, supplied by Oxford Labware) can be used. For example, fixed, dehydrated tissue can be transferred from the second xylene wash to paraffin or a paraffin/polymer resin in the liquid-phase at about 58°C, then replace three to six times over a period of approximately three hours to dilute out residual xylene, followed by overnight incubation at 58°C under a vacuum, in order to optimize infiltration of the embedding medium in to the tissue. The next day, following several more changes of medium at 20 minute to one hour intervals, also at 58°C, the tissue sample is positioned in a sectioning mold, the mold is surrounded by ice water and the medium is allowed to harden. Sections of 6μm thickness are taken and affixed to 'subbed' slides, which are those coated with a proteinaceous substrate material, usually bovine serum albumin (BSA), to promote adhesion. Other methods of fixation and embedding are also applicable for use according to the methods of the invention; examples of these can be found in Humason, G.L., 1979, Animal Tissue Techniques, 4th ed. (W.H. Freeman & Co., San Francisco).

An available preparation of polyclonal antibodies against rat p130^{Cas} (Sakai et al., 1994, EMBO J., 13(16): 3748-3756) is known to cross-react with the human homologue of that protein, which we know to be BCAR1, with sufficient specificity to perform an immunohistochemical analysis of human cells in tissue culture (Black and Bliska, 1997, EMBO J., 16(10): 2730-2744). Antibodies directed against one or more of the BCAR1, BCAR2 and BCAR3 proteins are raised by methods well known in the art and used for immunostaining of the fixed tissue slices by standard methods (see Current Protocols in Immunology, 1994, John Wiley & Sons), for example: After antibodies are obtained, slides bearing fixed tissue are rehydrated and blocked by incubating them for thirty minute intervals in a mixture of 1% powdered milk and 2% BSA (Fraction V) in phosphate buffered saline (PBS) with 0.05% Tween-20 two to four times. After blocking, slides are incubated with the primary antibody for one hour at room temperature or overnight at 4°C. This antibody can be either monoclonal or polyclonal, and, if the latter, can be either purified or used as a crude serum extract. In any case, appropriate concentrations of antibody must be determined for

each preparation. A typical crude antiserum raised in a mouse, rat or rabbit is diluted 1:2000 in PBS with 0.05% Tween-20 for use. Following incubation with the primary antibody, slides are washed in the same buffer, this time without antiserum, three or four times for 30 minutes each. An additional round of incubations in blocking solution is optional at this time, but is helpful in reducing non-specific binding of the secondary antibody.

After washing and/or re-blocking, slides are incubated with a secondary antibody directed at IgG molecules of the host organism in which the primary antibody was raised; for example, if a rabbit antiserum was used, one employs an anti-rabbit-IgG secondary antibody. Visualization of the bound primary/secondary antibody complexes can be performed in two ways. The secondary antibody can be complexed either to a fluorescent dye, such as fluorescein or rhodamine, or to an enzyme, such as horseradish peroxidase or alkaline phophatase, that will deposit a colored precipitate when incubated with a solution containing a chromogenic substrate. Both fluorescently-labelled and enzymatically-complexed secondary antibodies are commercially-available from numerous suppliers, including Vector Laboratories (Burlingame, CA) and Fisher Pharmaceuticals (Orangeburg, NY). The manufacturer's suggested concentration of each antibody should be used in each incubation, also in PBS/Tween-20 at room temperature, as above for 45 minutes to one hour at room temperature or overnight at 4°C. Again, following incubation with the antibody, slides are washed in PBS/Tween-20 without antibody several times at room temperature, for a total of 1 to 2 hours. If fluorescent dyes are used, bound complexes can be visualized using either a standard fluorescent microscope or, optimally, a confocal microscope. If enzymatic indicators are used, slides should be incubated in the appropriate staining solution according to the manufacturer's literature, and then subjected to direct visual examination.

In either case, a change in intensity of fluorescence or staining of at least five-fold over that observed in neighboring cells or in normal control cells is indicative of abnormal expression of BCAR1, BCAR2 or BCAR3. Such a difference may be fifteen-fold, thirty-fold, 100-fold or 500-fold above or below the level observed in normal cells, and is indicative of malignant cells growth. In certain cases, in which the tumor is derived from an endocrine-dependent tissue, the difference is indicative of endocrine-independent cell growth. Specifically, where the tumor is derived from an estrogen-dependent tissue, such as breast, ovarian or endometrial tissue, such a difference is indicative of estrogen-independent cell growth.

EXAMPLE 7

Detection of BCAR1 protein in normal and malignant breast tissue.

In the following diagnostic assay, which is representative of an assay according to the invention, BCAR1 protein was detected in malignant but not in normal (non-malignant) breast tissue from a number of breast tissue samples. The assay was performed as follows, and the results are reported below.

Antibodies directed against rat p130CAS (Transduction Laboratories) cross react with the human BCAR1 protein. Lysates of transfectants of ZR-75-1 overexpressing the BCAR1 protein show a prominent band of approximately 116 kDa on Western blots probed with this antibody. To investigate the presence of the BCAR1 protein in breast tissue, we analysed a series of ten non-malignant and 118 malignant primary breast tissue specimen. Sections were cut from frozen tissue specimen and solubilized by boiling in Tris/SDS solution (10mM Tris pH=7.5 and 1% SDS). Protein was quantitated using the BCA Protein Assay (Pierce) and 5µg loaded onto a 6% polyacrylamide SDS gel. Electrophoretically separated proteins were transferred onto membrane (Hybond P, Amersham) in blotbuffer (Glycin 39mM, Tris 48mM, 0.0375% SDS and 20% methanol) using a semi-dry blotting system (OWL Scientific, Woburn, MA, #HEP-1) for 1 hr at 1.2 mA/cm^2. Blots were pre-incubated with 0.6% BSA in TBS-T (Tris-buffered Saline plus 0.05% Tween-20) and subsequently incubated with 1/5000 diluted primary antibody for one hour. Standard washing procedures were applied (3 times TBS-T and 3 times TBS, for 10 minutes each) and blots were incubated for one hour with horseradish peroxidase-coupled rabbit-anti-mouse antibody (DAKO, Denmark. 1/10,000 dilution in TBS plus 1% non-fat milk). Antibody complexes were visualized with the ECL detection reagent (Amersham) on autoradiography film using different exposure times (30 seconds to 15 minutes).

The analyses demonstrate that expression of BCAR1 protein in non-malignant breast tissue was below the detection level of the assay. In contrast, malignant breast tissues showed variable levels of expression, ranging from absent to very high expression. This was confirmed by immunohistochemical analysis of breast tumor sections. Tumors with high expression of BCAR1 on western blots exhibited abundant staining in the malignant epithelial cells. Stromal cells were mostly negative in these sections.

In order to determine the role of BCAR1 in malignant progression of the disease and in tamoxifen response, a large series of primary breast tumors was analysed. Tumor

fractionation experiments indicated that the bulk of BCAR1 protein could be recovered in the cytosol fraction. This result allowed us to use the stored routine cytosol preparations, which had been used previously for steroid receptor determination and to test various other putative markers (Nooter *et al.*, Lancet 349: 1885-1886, 1997; Berns *et al.*, J. Clin. Oncol. 16: 121-127, 1998; Foekens *et al.*, J. Clin. Oncol. 16: 1013-1021, 1998). About 5 µg of cytosolic protein was separated on gel and transferred onto membrane and analysed as described above. As controls, total lysates of the transfectant overexpressing the BCAR1 protein and the parental ZR-75-1 cells were included in the analyses. Various exposures of the films were evaluated for BCAR1 expression levels. Samples lacking a clear signal at the position of the BCAR1 protein after long exposure of the Western blot were scored negative, whereas samples showing high expression following short exposure were scored high. Intermediate BCAR1 protein levels were just detectable on the short exposure, whereas low expression was assigned to samples showing only detectable signals after prolonged exposure. The Western blot scoring of all samples was performed independently by three different investigators and discrepancies in scoring were then discussed. The consensus score was subsequently combined with the clinical data of the 775 breast cancer patients (median follow-up of patients alive 105 months, range 18-175 months) and analysed by statistical methods as described by Foekens et al., 1998.

The expression of BCAR1 was undetectable in 33%, low in 39%, moderate in 20% and high in 8% of primary tumors of these patients. A high level of expression of BCAR1 protein was predominant in the ER-positive tumors and reduced in the EGFR-positive tumors. Both in univariate (Fig. 18) and in multivariate analysis including age/menopausal status, nodal status, tumor size and steroid-hormone receptor status, a high expression level of BCAR1 in primary breast tumors was associated with an increased rate of relapse (relative relapse rate=1.6; multivariate p-value=0.012). Interestingly, a high expression level of BCAR1 was associated with a poor response to first-line tamoxifen therapy in 282 patients with recurrent disease, also when corrected for age/menopausal status, disease-free interval, site of relapse, and steroid-hormone receptor status (odds ratio=0.35; multivariate p-value=0.037). Thus, overexpression of BCAR1 in the primary tumors is a marker of poor clinical behaviour of the disease.

USE

The invention is useful for the detection of malignant cells or in the monitoring of tumors for the transition to malignant cell growth. The invention is of further use in the detection of endocrine-independent cells and the monitoring of tumor cells for the transition to endocrine-independent cell growth. The invention is particularly useful for the detection of tumor cells that divide in an estrogen-independent manner, despite having arisen from a tissue that is normally depends on estrogen to stimulate cell proliferation and for the monitoring of tumor cells for the transition to estrogen-independent cell growth. Examples of estrogen-dependent tissues are those of the breast, ovary and endometrium. The invention is more useful for the detection of such estrogen-independent cells that are cancerous, since the presence of estrogen-independent cancer cells that are derived from cells that are normally estrogen-dependent is indicative that cancer cell growth will be antiestrogen-resistant. In particular, the invention is useful to monitor breast cancer for the presence of estrogen-independent cancer cells, the appearance of which signals the transition from antiestrogen-sensitivity to a more aggressive and intractable antiestrogen-resistant cell-proliferative phenotype. Similarly, the invention is useful to monitor the progression of cancers of the ovary and endometrium.

OTHER EMBODIMENTS

Other embodiments will be evident to those of skill in the art. It should be understood that the foregoing detailed description is provided for clarity only and is merely exemplary. The spirit and scope of the present invention are not limited to the above examples, but are encompassed by the following claims.

CLAIMS

1. A method for the diagnosis or prognosis of malignant cell growth, which method comprises measuring the expression of one or more of the *BCAR1, BCAR2* or *BCAR3* genes in a biological sample from a patient.

2. A method according to claim 1 which comprises measuring the expression of a panel of genes which panel comprises one or more of a first gene of *BCAR1, BCAR2* or *BCAR3* in combination with one or more of a second gene of *ER, EGFR, PR, RAS, ILGF-II, HER2/NEU, FGF-4, TGFβ1* and *Cyclin D1* in a biological sample from a patient and comparing the ratio of expression levels of said first and second genes in said sample to the ratio of expression levels of said first and second genes observed in normal tissue.

3. A method according to claim 1 or 2 wherein detection of expression of one or more of the *BCAR1, BCAR2* or *BCAR3* genes is elevated in said sample in comparison to normal tissue.

4. A method according to any one of the preceding claims for the detection of a malignant cell.

5. A method according to any one of claims 1 to 3 for monitoring the malignancy of tumor cell growth.

6. A method according to any one of claims 1 to 3 for detecting an estrogen-independent breast tumor cell.

7. A method according to any one of claims 1 to 3 for monitoring the estrogen independence of breast tumor cell growth.

8. A method according to any one of the preceding claims, further comprising the step of obtaining a result wherein said one or more *BCAR1, BCAR2* or *BCAR3* genes is expressed at a level in said sample that is elevated in comparison to that observed in normal tissue,

wherein elevated expression is indicative of a malignant or tumor cell.

9. The method of claim 8, wherein said malignant or tumor cell is antiestrogen-resistant.

10. A method according to claim 8 or 9, wherein said normal tissue is of the same tissue type from which said malignant cell or tumor has arisen.

11. A method according to any one of the preceding claims, wherein said biological sample comprises tumor cells.

12. A method according to claim 11, wherein said tumor cells have arisen from breast tissue.

13. A method according to any one of the preceding claims, wherein said measuring comprises contacting the protein of said sample with an antibody directed at the protein product of a *BCAR1, BCAR2* or *BCAR3* gene and performing a detection step for said antibody complexed to said product or to a portion or a fragment thereof.

14. A method according to claim 13, wherein said measuring comprises an ELISA.

15. A method according to claim 13, wherein said measuring comprises a Western blot or immunostaining of fixed cells of said sample.

16. A method according to any one of claims 1 to 12 wherein said measuring comprises detection of nucleic acid of said sample.

17. A method according to claim 16, wherein said nucleic acid is mRNA.

18. A method according to claim 17, wherein said measuring comprises performing hybridization of a labelled, nucleic acid probe specific to the complement to a sequence of a *BCAR1, BCAR2* or *BCAR3* gene to said mRNA and a step to detect hybridized complexes of said probe to the mRNA product of said gene or to a portion or a fragment thereof.

19. A method according to claim 17 or 18, wherein said hybridization is performed on a Northern blot.

20. An isolated nucleic acid which comprises SEQ ID NO:1 or a fragment thereof at least 10 nucleotides in length that is specific for SEQ ID NO:1.

21. An isolated nucleic acid which comprises any one of SEQ ID NOs:7-19 or a fragment thereof at least 10 nucleotides in length that is specific for *BCAR1*.

22. An isolated nucleic acid which comprises SEQ ID NO:3 or a variant having at least 70% homology thereto, or a fragment of said nucleic acid or variant at least 10 nucleotides in length that is specific for SEQ ID NO:3.

23. A fragment according to claim 22 which comprises nucleotides 39 to 3042 of SEQ ID NO:3 or a fragment thereof.

24. An isolated nucleic acid which comprises SEQ ID NO:20, SEQ ID NO:22 or SEQ ID NO:24 or variants thereof having at least 70% homology thereto, or a fragment thereof at least 10 nucleotides in length that is specific for SEQ ID NO:20, SEQ ID NO:22 or SEQ ID NO:24.

25. Use of a nucleic acid specific for any one of the sequences SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7-19, SEQ ID NO:20, SEQ ID NO:22 or SEQ ID NO:24 in a method according to any one of claims 1 to 12 or 16 to 19.

26. An isolated polypeptide which comprises a SEQ ID NO:2, or a fragment thereof retaining an epitope specific for *BCAR1*.

27. An isolated polypeptide which has at least 70% identity to SEQ ID NO:4, or a fragment thereof retaining an epitope specific for *BCAR3*.

28. An isolated polypeptide which has at least 70% identity to any one of SEQ ID

NO:21, SEQ ID NO:23 or SEQ ID NO:25, or a fragment thereof retaining an epitope specific for *BCAR2*.

29. An antibody specific for a polypeptide of claim 27 or 28.

30. Use of an antibody specific for any one of the sequences SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:21, SEQ ID NO:23 or SEQ ID NO:25 in a method according to any one of claims 1 to 15.

31. A kit for the detection of the mRNA product of a *BCAR1, BCAR2* or *BCAR3* gene which comprises a nucleic acid probe at least 10 nucleotides in length that is specific for any one of the sequences SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7-19, SEQ ID NO:20, SEQ ID NO:22 or SEQ ID NO:24.

32. A planar array of RNA samples derived from breast tissue affixed to the surface of a solid support matrix and probed with a labelled nucleic acid probe at least 10 nucleotides in length that is specific for sequences encompassed by SEQ ID NO: 1, 3, 20, 22 or 24.

33. The array of claim 32, wherein said planar array is one of a Northern blot or an RNA dot blot.

34. An autoradiographic film that has been exposed to the planar array of claim 33 for a time and under conditions sufficient to permit detection of bound probe.

35. A digital, photographic, xerographic or chart record of the planar array of claim 33 that contains evidence of bound probe that is labelled luminescently, radioactively or with a visible chemical precipitate on said support matrix.

36. An autoradiographic film that has been exposed to a sample of cells to which a labelled nucleic acid probe at least 10 nucleotides in length that is specific for sequences encompassed by SEQ ID NO: 1, 3, 20, 22 or 24 has been hybridized for a time and under conditions sufficient to permit detection of bound probe.

37. An autoradiographic film that has been exposed to a sample of cells to which is bound a labelled primary antibody directed against one or more of the proteins BCAR1, BCAR2 and BCAR3, for a time and under conditions sufficient to permit detection of the bound, labelled antibody.

38. A digital, photographic, xerographic or chart record of the cells of claim 37 that documents the presence on said cells of bound, labelled antibody.

39. An autoradiographic film that has been exposed to a sample of cells to which is bound,

(a) a primary antibody directed against one or more of the proteins BCAR1, BCAR2 and BCAR3, and

(b) a labelled secondary antibody directed against said primary antibody, for a time and under conditions sufficient to permit detection of the bound, labelled secondary antibody.

40. A digital, photographic, xerographic or chart record of the cells of claim 39 that documents the presence on said cells of bound, labelled secondary antibody.

41. An autoradiographic, digital, photographic, xerographic or chart record of a planar array of proteins on the surface of a solid support matrix to which is bound

(a) a primary antibody directed against one or more of the proteins BCAR1, BCAR2 and BCAR3, and

(b) a labelled secondary antibody directed against said primary antibody, that documents the presence on said matrix of bound, labelled secondary antibody.

CORRECTED VERSION*

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(54) Title: BREAST CANCER RESISTANCE GENES, METHODS FOR THEIR DETECTION AND USES THEREOF

(57) Abstract

The present invention relates to the finding of genes associated with the development of estrogen independent malignant cell growth, particularly of breast cancer cells. The invention provides assay methods for the diagnosis or prognosis of malignant cell growth, which method comprises measuring the expression of one or more of the *BCAR1*, *BCAR2* or *BCAR3* genes in a biological sample from a patient. Measurement may be by immunological techniques or by hybridisation with nucleic acids, utilising novel sequences and antibodies of the invention.

*(Referred to in PCT Gazette No. 30/1999, Section II)

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INTERNATIONAL SEARCH REPORT

Inter ›nal Application No
PCT/EP 98/04754

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/574

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DORSSERS, L. C. J. ET AL: "Identification and cloning of a human breast cancer antiestrogen resistance locus (bcar1) by provirus insertion mutagenesis." PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, (1993) VOL. 34, NO. 0, PP. 497. MEETING INFO.: 84TH ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ORLANDO, FLORIDA, USA MAY 19-22, 1993 ISSN: 0197-016X., XP002091638 see abstract --- -/--	20,21, 25,26,30

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search	Date of mailing of the international search report
29 January 1999	21. 06. 1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hoekstra, S

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/EP 98/04754

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VAN DER FLIER S ET AL: "Identification of the gene within the bcar1 locus causing antiestrogen resistance in human breast cancer cells in vitro (Meeting abstract)." PROC ANNU MEET AM ASSOC CANCER RES, (1996). VOL. 37, PP. A1596., XP002091639 see abstract	20,21, 25,26,30
A	VAN AGTHOVEN, T. ET AL.,: "Ectopic expression of epidermal growth factor receptors induces hormone independence in zr-75-1 human breast cancer cells." CANCER RESEARCH, vol. 52, 1992, pages 5082-5088, XP002091640 cited in the application see the whole document	1-31
A	US 5 292 638 A (BENZ CHRISTOPHER C ET AL) 8 March 1994 see the whole document	1-31
A	WO 92 03973 A (UNIV TEXAS ;UNIV YALE (US)) 19 March 1992 see the whole document	1-31
A	DORSSERS, L.C.J. ET AL.,: "Induction of antiestrogen resistance in human breast cancer cells by random insertional mutagenesis using defective retroviruses: Identification of bcar-1, a common integration site." MOLECULAR ENDOCRINOLOGY, vol. 7, no. 7, - 1993 page 870-878 XP002091641 see the whole document	1-31

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. ational application No.
PCT/EP 98/04754

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 32-41
beoause they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-19,25,30,31(partially); 20,21,26(completely)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 20,21,26 entirely and 1-19,25,30,31 partially

   BCAR1 and methods involving BCAR1

2. Claims: 24,28 entirely and 1-19,25,29,30,31 partially

   BCAR2 and methods involving BCAR2

3. Claims: 22,23,27 entirely and 1-19,25,29,30,31 partially

   BCAR3 and methods involving BCAR3

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claims Nos.: 32-41

In claim 32 a product ("a planar array") is claimed which is itself not characterisd by technical features. It is only characterised by it being probed by an undefined probe. Not that the probe is not defined by those technical (sequence) features that allows one to test if a probe derived from SEQ ID NO's 1, 3, 20, 22 or 24 with the length limitations is indeed specific for SEQ ID NO's 1, 3, 20, 22 and 24.

Claims 36, 37, 39 and 41 go one step further and the same holds true mutatis mutandis.

Claim 32 and 36, 37, 39 and 41 and claims referring thereto do in consequence not meet the prescribed requirements (Articles 5, 6 PCT, Rule 5, 6 PCT) to such an extent that a meaningful search is not possible.

INTERNATIONAL SEARCH REPORT

..formation on patent family members

Inte ional Application No
PCT/EP 98/04754

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5292638 A	08-03-1994	NONE	
WO 9203973 A	19-03-1992	US 5119827 A	09-06-1992
		CA 2089162 A	06-03-1992
		EP 0547141 A	23-06-1993
		US 5384260 A	24-01-1995